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[Continued on next page]

(54) Title: METHODS AND COMPOSITIONS USEFUL FOR STIMULATING AN IMMUNE RESPONSE

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RhUS28.1

        10         20         30         40         50         60
ATGAATAACA CATCTTGCAA CTTCAACGTC ACTCTCAACG CATCGGCACC AAGCCGATAC

        70         80         90        100        110        120
ATAGCTATTG CTATGTACAG CATTGTTATC TGTATCGGGT TGGTTGGAAA CCTGCTGTTA

       130        140        150        160        170        180
TGCATCGTGT TAGTCAAGAA ACGCAAACTG CGATATTCCA GCGATGTTTA TTTTTTCCAC

       190        200        210        220        230        240
GCCTCTATGG CCGACCTCGT CAGCACTGTC ATGCTACCGC TCTGGCTACA TTATGTCCTC

       250        260        270        280        290        300
AACTTTGCCC AACTCTCTCG AGGAGCCTGT ATCAGCTTTT CGGTGACTTT CTATGTTCCC

       310        320        330        340        350        360
CTTTTCGTTC AGGCCTGGTT ACTCATTTCC ATCGCTATGG AGCGATATTC CAACTTAGTA

       370        380        390        400        410        420
TGGATGGCAC CCATTAGCGT TAAGACGGCC TTTAAACACT GCATAGGAAC CTGGATCGTA

       430        440        450        460        470        480
TCTGCCTTCG TGGCATCACC CTACTACGCA TACAGAAACT CACACGACGA ACACGAATGC

       490        500        510        520        530        540
ATTCTAGGAA ACTACACTTG GCACATTAAC GAACCGCTAC ACACGTGTAT GGATGTGGTG

       550        560        570        580        590        600
ATCATAGTAT GGACCTTTTT GGCCCCAGTA CTGGTAACCA TTATAGCAAG CGTCAAAATG

       610        620        630        640        650        660
AGACGAACGA CCTGGGGCAA TACTAGGTTA AACGAAAAGA ACAGCGACAT TCTTATAGTA

       670        680        690        700        710        720
CTAGTTGTCA TGACAGTGTT CTTTTGGGGA CCGTTTAATA TCGTGTTGGT TATTGACAAT

       730        740        750        760        770        780
ATTTTACAGA GATACTATGA TACCACGAAT TGCGATGTAG AAAAGATTAA ACATATCATG

       790        800        810        820        830        840
GCTATGATCT CAGAAGCCAT TGTTTATTTT CGCGGTATTA CAGCACCTAT TATTTATGTA

       850        860        870        880        890        900
GGGATTAGTG GCAGATTTCG CGAAGAGATT TACTCTCTGT TTAGACGCCA GCCGTATAAC

       910        920        930        940        950        960
GATTTGGACC CCGATGCCAA TCAATTCATG ATTGAACTCA CTAGCCAGGG AAGAAGTAGA

       970        980        990       1000       1010       1020
AATAGAAATG CTAGACAATC GGAAAGCAAT GTACCGCAAC CAGAAGAATG CTTCTGGTAA

      1030       1040       1050       1060       1070       1080
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(57) Abstract: The invention provides compositions containing modified cytomegalovirus, and methods of using the compositions. In various embodiments, the modifications include adding a heterologous sequence encoding a non-viral chemokine element and/or immunogenic polypeptide, and/or disabling a viral dissemination gene.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS AND COMPOSITIONS USEFUL FOR STIMULATING AN IMMUNE RESPONSE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/265,925, filed February 2, 2001, which is incorporated herein by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was partially made with government support under Grant Number N66001-01-C-8009 awarded by the Defense Advanced Research Projects Agency (DARPA) of the Department of Defense. The government may have certain rights in this invention.

BACKGROUND

Cytomegaloviruses (CMVs) are common pathogens and are members of the β subgroup of the herpesvirus family. CMV is a slow replicating, species-specific complex DNA virus found in most mammals. CMV has adopted subtle evolutionary strategies for evading the immune system of an infected host, while disseminating through the host tissues.

The genome (230 kb) of human CMV (HCMV) includes a long and short unique region (UL and US, respectively), each of which is flanked by inverted repetitions. The entire HCMV genome has been sequenced (Chee, M.S., *et al.* (1990) *Curr. Top. Microbiol. Immunol.* 154:125-169) and appears to contain over 200 open reading frames.

One of these open reading frames is referred to as US28, which encodes a protein (also "US28") that acts as a functional receptor for certain human and viral chemokines (see, e.g., Gao & Murphy, 1994, *J Biol Chem.* 269:28539-42). Upon infection of a cell by CMV, US28 is expressed on the surface of the infected cell and becomes capable of responding to chemokines in the environment. Three other open reading frames called US27, UL33 and UL78 encode for proteins having homology to US28 as shown in Table 1 below.

Table 1: Exemplary Viral Chemokine Elements

CMV Chemokine Elements	GenBank Accession No.	Reference
US27	X17403	Chee et al, 1990, *Nature*, 344:774
US28	L20501, AF073831-35	Neote et al, 1993, *Cell*, 72:415-25
UL33	X53293	Chee et al, 1990, *Nature*, 344:774
UL78	X17403	Chee et al, 1990, *Nature*, 344:774

Chemokine receptors such as US28 generally are G protein coupled receptors. Structurally these receptors have seven transmembrane segments that loop in and out of the cell membrane, as well as an intracellular tail that is coupled to a G protein signal transducing molecular complex.

The chemokines themselves constitute a subgroup of a larger class of signaling proteins and have the ability, among other things, to promote cellular migration (Zlotnik *et al.* (1999) *Crit. Rev. Immunol.* 19:1-47). The chemokines generally are divided into four groups based upon the arrangement of certain cysteine residues within the protein that can form disulfide bonds. One class of chemokines is the beta chemokines that are characterized by having two adjacent cysteines; this structure is referred to in shorthand form simply as CC. The beta chemokines are involved in attraction of monocytes and leukocytes. The alpha chemokines, in contrast, have a single amino acid separating the two cysteine residues, and thus their structure is designated as CXC. These chemokines are primarily involved in attracting polymorphonuclear cells. The fractalkines or delta-chemokines constitute a third class of chemokines and tend to be cell bound molecules. The two cysteines in this class are separated by three amino acid residues, a structure designated as CX3C. This class of chemokines are expressed at high levels in the brain; some evidence indicates that the fractalkines are involved in neuron-glial cell interactions (see, e.g., Harrison, *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:10896-10901; and Nishiyori, A. *et al.* (1998) *FEBS Lett.* 429:167-172). The US28 receptor of HCMV is characterized in part by its very strong affinity for fractalkine. The structure of the final class of chemokines is simply referred to as C (also gamma-chemokines), because these chemokines contain only a single N-terminal cysteine involved in a disulfide bond. The chemokine receptors have varying specificity for the different classes of chemokines. Some chemokine receptors can bind chemokines from different classes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the polynucleotide sequence of the rhUS28.1 coding sequence (SEQ ID NO:1).

Figure 2 shows the polynucleotide sequence of the rhUS28.2 coding sequence (SEQ ID NO:2).

Figure 3 shows the polynucleotide sequence of the rhUS28.3 coding sequence (SEQ ID NO:3).

Figure 4 shows the polynucleotide sequence of the rhUS28.4 coding sequence (SEQ ID NO:4).

Figures 5A-5B show the polynucleotide sequence of the rhUS28.5 coding sequence (SEQ ID NO:5).

Figure 6 shows the polynucleotide sequence of the rhUL33 coding sequence (SEQ ID NO:6).

Figures 7A-7B show the polynucleotide sequence of the rhUL33 spliced coding sequence (SEQ ID NO:7).

Figure 8 shows the polynucleotide sequence of the rhUL78 coding sequence (SEQ ID NO:8).

SUMMARY

Compositions containing recombinant or modified cytomegalovirus (CMV) are provided herein, as well as methods utilizing such compositions to generate an immune response and/or in various therapeutic or prophylactic treatments. Some of the recombinant CMV contain a targeting sequence that targets the recombinant CMV to the immune system cells and tissues of the host in which the composition is to be administered. Typically, the targeting sequence is a heterologous chemokine element, such as a chemokine or chemokine receptor. Other recombinant CMV incorporate a heterologous sequence that encodes for an immunogenic protein or peptide; such modified CMV can serve as a vehicle for delivering a selected antigen to a host to generate a desired immune response. Certain other modified CMV are attenuated by disabling a viral dissemination gene to reduce the virulency of the modified CMV in the host. Still other recombinant CMV incorporate some or all of the foregoing elements.

Thus, certain recombinant cytomegalovirus (CMV) comprise a cytomegalovirus (CMV) genome which comprises a first heterologous nucleotide sequence encoding a heterologous chemokine element (i.e., a targeting sequence), and (ii) a second heterologous nucleotide sequence encoding an immunogenic polypeptide. The recombinant

CMV is typically encapsulated to form an infectious and biologically active virus. The heterologous chemokine element is generally selected to be endogenous to the host in which the composition is to be administered, as this facilitates targeting of the recombinant CMV to the immune tissues of the host. As indicated *supra*, the heterologous chemokine element can be a chemokine or chemokine receptor. Specific examples of such elements useful when the host is a mammal include, but are not limited to, MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or homolog thereof.

The heterologous nucleotide sequence that encodes the immunogenic polypeptide is typically a sequence that encodes an antigen from a pathogenic organism or for a tumor antigen, but this is not required. The pathogenic organism can be a bacterium, virus, or parasite, for example.

Some of these recombinant CMV are further modified such that the CMV is attenuated to reduce virulency in a host. As noted *supra*, the CMV genome can be attenuated in various ways, such as disabling a viral dissemination gene. Such genes can be those that encode a viral chemokine element or a viral immune-modulatory gene. The viral chemokine elements can be a chemokine, chemokine receptor or a soluble chemokine binding protein, for example. Specific examples of such chemokines and chemokine receptors include, for example, US28, US27, UL33, UL78, UL146, UL147, MCK-1 and MCK-2, or a homolog thereof. The viral immune-modulatory genes are those viral genes that modulate the anti-viral immune response of an infected host so as to facilitate viral infection. Specific examples of such genes include, but are not limited to, UL111A, US3, US6, US11, US2, UL83, UL18, UL40, m144, m152, m04, m06, and m138, or a homolog thereof.

The foregoing recombinant CMV can optionally be formulated as a composition. Such compositions then contain recombinant CMV and a pharmaceutically acceptable adjuvant, carrier, diluent or excipient.

Recombinant CMV such as just described can be utilized in methods to induce an immune response in a host. In general such methods involve administering a composition to a host, wherein the composition comprises a recombinant cytomegalovirus (CMV) with a genome that contains (i) a first heterologous nucleotide sequence encoding a heterologous chemokine element, and (ii) a second heterologous nucleotide sequence encoding an immunogenic polypeptide. The heterologous chemokine element and immunogenic polypeptide are as just described for the recombinant CMV compositions and reagents. The recombinant CMV may also be attenuated by disabling one or more viral dissemination genes as described *supra*. In some instances, the nucleotide sequence that encodes the

immunogenic polypeptide is selected to encode an antigen correlated with a disease or infection which the host has or is susceptible to obtaining. Such is the case for therapeutic and prophylactic treatment methods which are discussed below. However, the sequence need not encode for such an antigen. This is sometimes the case when a study is performed with an animal model (e.g., rhesus monkey or mice). For instance, a study may be under taken in rhesus monkeys or mice to make a preliminary assessment of the effectiveness of a recombinant CMV encoding for an antigen that appears to be correlated with a human disease.

As just alluded to, the recombinant CMV that are provided are also useful in treatment methods, either therapeutically or prophylactically. Some of these methods involve administering a composition to an animal, wherein the composition comprises an attenuated recombinant cytomegalovirus (CMV) with a genome that contains (i) a first nucleotide sequence encoding a chemokine receptor or chemokine that is endogenous to the animal, and (ii) a second nucleotide sequence encoding an immunogenic polypeptide. Because the immunogenic polypeptide comprises an antigen correlated with a disease or infection which the animal has or is susceptible to obtaining, and the administered composition induces an immune response in the animal, the method provides effective therapeutic or prophylactic treatment.

DETAILED DESCRIPTION

I. Definitions

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); THE GLOSSARY OF GENETICS, 5TH ED., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991).

The following definitions are provided to assist the reader in the practice of the invention.

As used herein, the term "cytomegalovirus (CMV)" has the normal meaning in the art and refers to one of a family of double stranded DNA viruses of the betaherpes group with positional and genomic similarity to human herpes virus 5 (cytomegalovirus) including, without limitation, human CMV AD169 (ATCC # VR 538), human CMV Towne (ATCC # VR 977), human CMV Davis (ATCC # VR 807), human CMV Toledo (Quinnan et al, 1984, *Ann Intern Med* 101: 478-83), monkey CMV Rh68.1 (ATCC # VR 677), monkey CMV CSG (ATCC # VR 706), rat CMV Priscott (ATCC # VR 991), mouse CMV Smith (ATCC # VR 1399) and others. "ATCC" is the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA. The 230-kb dsDNA genome of human and murine CMV were sequenced (see, e.g., Chee et al., 1990, *Curr. Top. Microbiol. Immunol.* 154:125-169; also see Rawlinson, 1996, *J Virol.* 70:8833-49, both incorporated herein in their entirety).

Various open reading frames from human CMV (HCMV) are referred to herein using the nomenclature of Chee *et al* [e.g., US28, US33, US78 (human US28, human US33, human US78, respectively)]. In general, reference to such reading frames from HCMV also refer to the sequences of sequence and positional homologs of such reading frames found in different HCMV strains, including sequences in any naturally occurring HCMV strain, and mutations to such strains. In some instances the term can also refer to various splice variants not yet characterized in the literature. With respect to the protein, the protein encoded by the HCMV reading frame refers to the protein having a native amino acid sequence, as well as variants and fragments regardless of origin or mode of preparation.

As used herein the term homolog has its usual meaning in the art. The term generally refers to a nucleic acid or protein that has substantial sequence identity with respect to a reference sequence and, in the case of a protein, typically shares at least one activity with the reference sequence. Thus, for example, the term "US28 homolog" refers to a nucleic acid or protein that has sequence homology with US28 and at least one activity of US28, typically the ability to bind a chemokine, especially fractalkine. The US28 homolog can be from CMV native to various animals, including various mammals (e.g., human and non-human primates, specifically monkeys, chimpanzee, gorilla and baboon). Thus, US28 homologs can include, but are not limited to, human US27, human UL33, and human UL78. Additional homologs from rhesus monkey (macaca mulatta) CMV can include rhUS28.1, rhUS28.2, rhUS28.3, rhUS28.4, rhUS28.5, rhUL33 and rhUL78.

As used herein, the term "immunogen" has the normal meaning in the art and refers to a molecule that can elicit an adaptive immune response upon injection or delivery by another mode into a person or animal, typically a peptide, polypeptide, glycoprotein, lipopolysaccharide or glycosaminoglycan. An "immunogenic polypeptide" is a polypeptide that is an immunogen.

As used herein, the term "antigen" has the normal meaning in the art and refers to a molecule that reacts with antibodies or elicits an immune response.

As used herein, the term "antigen presenting cell (APC)" has the normal meaning in the art and refers to a cell that can present antigen in the context of MHC I or MHC II to efficiently stimulate immune effector or helper cells (e.g., dendritic cells and macrophages).

As used herein, the term "resident dendritic cell (RDC)" has the normal meaning in the art and refers to a subclass of dendritic cells that, in the unstimulated state, are resident in peripheral tissue or organs rather than migratory. An exemplary RDC is a Langerhan cell, which is resident in skin.

As used herein, the term "secondary lymphoid organs" has the normal meaning in the art. Secondary lymphoid organs include lymph nodes, spleen and mucosal-associated lymphoid tissues such as tonsils and Peyer's patches.

As used herein, the term "chemokine element" refers to a chemokine or chemokine receptor or other (e.g., soluble) chemokine binding protein.

As used herein, the terms "chemokine" and "chemokine receptor" have their normal meanings in the art. Chemokines are a class of cytokines that play an important role in inflammatory responses, leukocyte trafficking, angiogenesis, and other biological processes related to the migration and activation of cells. As mediators of chemotaxis and inflammation, chemokines play roles in pathological conditions. As described in the Background section, known chemokines are typically assigned to one of four subfamilies based on the arrangement of cysteine motifs and include: the alpha-chemokines, the beta-chemokines, the gamma chemokines and the delta- chemokines. For a recent review on

chemokines, see Ward et al., 1998, *Immunity* 9:1-11 and Baggiolini et al., 1998, *Nature* 392:565-568, and the references cited therein. Chemokine activity may be mediated by chemokine receptors. For example, several seven-transmembrane-domain G protein-coupled receptors for C-C chemokines have been cloned: a C-C chemokine receptor-1 which recognizes MIP-1α, RANTES, MCP-2, MCP-3, and MIP-5 (Neote *et al.*, 1993, *Cell*, 72:415-415); CCR2 which is a receptor for MCP1, 2, 3 and 4 or 5; CCR3 which is a receptor for RANTES, MCP-2, 3, 4, MIP-5 and eotaxin; CCR5 which is a receptor for MIP-1α, MIP-1β and RANTES; CCR4 which is a receptor for CMDC or TARC; CCR6 which is a receptor for LARC; and CCR7 which is a receptor for SLC and MIP-3β (reviewed in Sallusto et al., 1998, *Immunol. Today* 19:568 and Ward et al., 1998, *Immunity* 9:1-11).

The terms "nucleic acid" "polynucleotide" and "oligonucleotide" are used interchangeably herein and refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof. A "subsequence" or "segment" refers to a sequence of nucleotides that comprise a part of a longer sequence of nucleotides.

A "primer" is a single-stranded polynucleotide capable of acting as a point of initiation of template-directed DNA synthesis under appropriate conditions (*i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization, such as, DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer but typically is at least 7 nucleotides long and, more typically range from 10 to 30 nucleotides in length. Other primers can be somewhat longer such as 30 to 50 nucleotides long. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. The term "primer site" or "primer binding site" refers to the segment of the target DNA to which a primer hybridizes.

As used herein, a "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, that has control elements that are capable of affecting expression of a gene that is operably linked to the control elements in hosts compatible with such sequences. Expression cassettes typically include at least promoters and optionally, transcription termination signals and polyadenylation signals. Typically, the recombinant expression cassette includes at least a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a non-viral chemokine or antigenic peptide sequence) and a promoter. Additional factors necessary or helpful in effecting expression can also be used as described herein. For example, transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

As used herein, the term "host" refers to an animal, e.g., a mammal such as a rodent, mouse, monkey, human or non-human primate, model animals, or agriculturally important livestock such as bovines, porcines, poultry, that can be infected by naturally occurring CMV or recombinant CMV as described herein.

As used herein, an "immune response" has the ordinary meaning in the art and, unless otherwise specified, refers to an adaptive immune response to a specific antigen. In one aspect, an immune response involves the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, and various soluble macromolecules in defending the body against infection, cancer or other exposure to non-self molecules. The immune response can be detected and quantified (e.g., following immunization) by measuring cellular or humoral responses according to numerous assays known in the art (see, e.g., Coligan et al., 1991 (suppl. 1999), CURRENT PROTOCOLS IN IMMUNOLOGY, John Wiley & Sons (hereinafter, sometimes "Coligan")). For example, to detect a cellular immune response, T cell effector effects against cells expressing the antigen are detected using standard assays, e.g., target-cell killing, lymphocyte proliferation, macrophage activation, B-cell activation or lymphokine production. Humoral responses are measured by detecting the appearance of, or increase in titer of, antigen-specific antibodies using routine methods such as ELISA. The progress of the antibody response can be determined by measuring class switching (i.e., the switch from an early IgM response to a later IgG response.

As used herein, "viral dissemination" has the normal meaning in the art, and refers to a detectable increase in viral titer or amount at sites other than the primary infection (inoculation) site, e.g., by transmission of virus from sites of primary infection or reactivation to secondary sites (e.g., tissues or organs). Virus dissemination typically involves transmission of virus from sites of primary infection (e.g., mucosal tissues such as oral or genital mucosal endothelia) or reactivation (e.g., blood leukocytes including myeloid progenitor cells in the bone marrow and peripheral blood monocytes) to secondary sites (e.g., tissues or organs including salivary glands, kidney, spleen, liver and lungs) where viral replication and amplification may occur. Without intending to be bound by a particular mechanism, dissemination may involve assisted movement of virus from primary sites (e.g., by random or directed migration of infected cells), release of virus into the bloodstream and random or directed attachment of this virus to cells at secondary sites, or other modes.

As used herein, the term "substantial sequence identity," refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90%, 95%, 98%, or 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. "Moderate sequence identity" refers to two or more sequences or subsequences that have at least 25% sequence identity, sometimes at least 28% or 30% sequence identity. Sequence identity that is less than substantial sequence identity can be significant, i.e., can be indicative of homology between molecules when other factors the (e.g., conserved motifs, functional similarity or structural similarity) are present, or, in the case of proteins, when a number of non-identical residues are similar (i.e., conserved). Two sequences (amino acid or nucleotide) can be compared over their full-length (e.g., the length of the shorter of the two, if they are of substantially different lengths) or over a subsequence such as at least about 50, about 100, about 200, about 500 or about 1000 contiguous nucleotides or at least about 10, about 20, about 30, about 50 or about 100 contiguous amino acid residues. For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology

algorithm of Smith & Waterman, 1981, *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman & Wunsch, 1970, *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al., supra). Each of these references and algorithms is incorporated by reference herein in its entirety. When using any of the aforementioned algorithms, the default parameters for "Window" length, gap penalty, etc., are used. One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., 1990, *J. Mol. Biol.* 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, 1989, *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

As used herein, the term "immune-inhibitory protein" or "immune modulatory protein" refers to CMV-encoded proteins that are believed to suppress or otherwise modify a host immune response to viral infection. An "immune-inhibitory gene" or "an immune modulatory gene" are genes that encode such proteins.

As used herein, an "endogenous" gene, polynucleotide, or polypeptide sequence is a gene, polynucleotide, or polypeptide sequence naturally occurring in a specified organism. For example, for CMV, an endogenous chemokine gene is a chemokine encoded by a wildtype CMV. Conversely, an "exogenous" sequence is one not naturally occurring in a specified organism. For example, for CMV, an gene sequence encoding a human chemokine is an exogenous sequence.

A "heterologous sequence" or a "heterologous nucleic acid," or a "heterologous polypeptide sequence" as used herein, is one that originates from a source foreign to the particular host cell or genome. Thus, in a virus genome, a synthetic or mammalian (e.g., rodent, Murine, bovine, porcine, human, non-human primate) gene sequence (e.g., such as a gene encoding a mammalian chemokine or chemokine receptor) is heterologous.

An "immunogenic amount" of a compound, agent or composition is an amount sufficient to induce an immune response in a host animal when administered to the host.

As used herein in the context of a gene sequence (e.g., in a CMV genome), the term "disabled" refers to a gene that is mutated, deleted or partially deleted in a coding or regulatory (e.g., promoter) sequence, such that the gene product (e.g., protein or RNA) that is encoded by the gene is not expressed or is not expressed in biologically active form.

As used herein, the term "peptide" refers to short (e.g., less than about 25-residues) polypeptides. Peptides are typically at least 6 residues in length, often at least 7 or eight residues. The term "polypeptide" refers polymers of amino acids and is includes peptides. In some embodiments, polypeptides are from about at least 6, 8, 10, or at least 25 residues up to about 100, 200, 500, 1000 or 2000 residues, e.g., between about 6 and about 500 residues, often between about 6 and about 200 or about 6 and about 100 residues. In some embodiments, polypeptides are from about 25 to about 100, about 25 to about 200, or about 25 to about 500 residues in length.

II. Introduction

Novel methods and reagents useful for inducing an immune response in a host to which the reagents are administered are provided herein. These reagents and methods include and utilize cytomegalovirus (CMV) that is genetically engineered to effectively deliver antigens (viral or exogenous) to immune system cells and tissues in the host (e.g., by altering the trafficking of cells that are infected with the genetically engineered virus and/or by altering the antigenic or immunogenetic potential of the virus).

In general this is accomplished by modifying the viral genome such that: (1) CMV is attenuated (i.e., rendered less pathogenic than wild type virus), typically by mutating (e.g., deleting) a gene that affects viral dissemination; and/or (2) the viral genome expresses a specified or predetermined antigenic or immunogenic polypeptide sequence to which an immune response is desired; and/or (3) the viral genome expresses a targeting element (e.g., a heterologous chemokine element such as a chemokine receptor or ligand) that effectively targets the virus to host immune system cells and tissues (e.g., professional antigen presenting cells or secondary lymphoid organs), resulting in an accelerated and/or more potent immune response against the antigen compared to conventional vaccination methods. It is contemplated that one, two or more of these changes are introduced.

The CMV that is modified can be a wild-type strain, a clinical strain, an attenuated strain, and/or a genetically engineered strain (e.g., comprising mutations other than those specifically introduced to accomplish the mutations described herein). Furthermore, the recombinant CMV disclosed herein is not limited to human CMV (HCMV). Similar recombinant CMV can be obtained for essentially any strain that infects animals, including humans, non-human primates and various commercial livestock. Specific examples of such modified CMV include rhesus monkey CMV (rhCMV) and murine CMV (mCMV) that incorporate the modifications disclosed herein.

The recombinant CMV can exist as a naked polynucleotide, but in the methods disclosed herein is generally encapsulated to form an infectious and biologically active virus. Furthermore, the recombinant CMV provided need not include the entire genome. As already indicated, certain genes can be deleted to attenuate the virus. Other genes can also be deleted or modified, provided the resulting virus is still capable of infecting the desired host or replicating and/or disseminating to the degree necessary to elicit an immune response.

Attenuation of the modified CMV is typically achieved by disabling one or more viral "dissemination genes." A dissemination gene is generally a gene that encodes a

chemokine element (e.g., a chemokine receptor, chemokine or chemokine binding protein) or a gene that plays a role in immune-modulation in the host. The nucleotide sequence encoding the immunogenic polypeptide is selected to encode at least an antigen to which one wants to raise antibodies. Typically, the antigen is one expressed by a pathogenic organism (e.g., a virus, a bacterium or a parasite) or a tumor antigen. The targeting element is generally a chemokine element that is endogenous to the host to facilitate delivery of the virus to the desired immune tissues or secondary lymphoid organs in the host. Often the chemokine element is a chemokine receptor or ligand that is expressed in the host.

Certain recombinant CMV provided herein include all three of the foregoing modifications and alter the activity of the virus in two ways. First, inclusion of a nucleotide sequence that encodes for an immunogenic protein (or fragment or variant thereof) results in expression of an immunogen which elicits an immune response in the host. Second, attenuation of the virus and modification of the CMV genome to include a nucleotide targeting segment collectively function to alter the dissemination pattern of the virus. As indicated *supra*, in certain embodiments CMV is attenuated to reduce virulency in the host by disabling a viral gene that functions in viral dissemination such that the encoded viral protein is not expressed in active form. Without intending to be bound by a particular mechanism, this modification impairs 'wild-type' CMV dissemination (which favors the virus establishing latent infection and may enhance pathogenicity). While the resulting virus is attenuated, it nonetheless serves as a vector for delivery of the encoded immunogenic polypeptide in the host. Engineering CMV to express a targeting element such as at least one non-viral (heterologous) chemokine element acts to target the virus to immune system tissues and cells in the host animal. This results in a change in the viral dissemination pattern from that of wild-type virus, which favors the virus establishing a latent infection, to a pattern that favors dissemination to specified host immune system cells and tissues. These changes result in decreased viral pathogenesis (as a consequence of inhibition of the wild-type dissemination pattern) and increased immune response to the antigen (as a consequence of dissemination of the virus to host lymphoid cells capable of initiating an immune response). In some instances, the modified CMV is as just described but is not attenuated. Such modified CMV finds utility in certain applications for targeting a specific immunogen to lymphoid tissue in a host.

Other modified CMV provided herein include one or two of the three modifications listed above. Thus, for instance, certain modified CMV are engineered to include just the sequence that encodes for an immunogenic polypeptide; optionally, such

modified CMV is also attenuated. Such modified CMV can be utilized to generate an immune response in the host. Other modified CMV are engineered to encode for a targeting element such as a chemokine receptor or ligand, and optionally are also attenuated as discussed above. Such modified virus is useful for targeting CMV bearing additional heterologous elements to specific immune tissues in a host.

In still other instances, the viral genome is modified to encode at least one non-viral (heterologous) chemokine element, and optionally at least one viral dissemination gene normally present in the wild-type virus is disabled, but the immunogen is a viral protein (i.e., expressed by wild-type CMV). Compositions containing such modified viruses are used to stimulate an adaptive immune response to CMV itself. In an embodiment, the viral genome is engineered to over-express the immunogenic viral polypeptide, or to express an immunogenic variant of the viral polypeptide.

The modified CMV compositions that are provided can be utilized to generate an immune response in a variety of hosts including humans and non-humans, including, for example, non-human primates such as rhesus monkeys and other mammals such as mice and rats. The generation of such immune responses can be utilized in diverse therapeutic and prophylactic treatment methods by facilitating the generation of antibodies against a particular antigen associated with a disease or infection, for example. Methods performed with non-humans can serve as models for human responses.

The following sections provide further details regarding certain aspects of the compositions and methods that are provided, as well as specific examples of such compositions and methods.

3. CMV Chemokine Element and Immune-Modulatory Genes

3.1 Viral Dissemination and Dissemination Genes

Cytomegaloviruses disseminate (move from the site of infection or latency to other sites in the host) following infection or reactivation. Although free virus particles (virions) can disseminate, dissemination is more often cell-mediated. That is, following infection of a cell by the virus, viral proteins are expressed by the cell and may be secreted or displayed on the cell surface. The expression of these viral proteins affects trafficking of the infected cells. Evidence indicates that dissemination of wild-type CMV is facilitated by several virus-encoded genes. Viral genes that encode proteins that function to promote viral dissemination are called "viral dissemination genes." In certain modified CMV provided herein, the pathogenic potential of CMV is attenuated by modification (e.g., disabling) of one

or more CMV dissemination genes, thereby increasing the efficacy of the virus for delivery of immunogen to host immune system cells and tissues by decreasing pathogenicity.

Two classes of genes play important roles in viral dissemination; in certain of the modified CMV, the virus is attenuated by disabling genes from one or both of these classes. The first class of viral dissemination genes is a class of genes encoding "chemokine elements," i.e., chemokines and chemokine receptors or soluble chemokine binding proteins. As noted *supra*, chemokines are proteins that mediate cell migration and targeting. Virally-encoded chemokine elements facilitate viral dissemination by mechanisms that include, without limitation, affecting the movement and targeting of virally-infected cells (e.g., cells secreting and/or displaying viral chemokines and/or chemokine receptors) and calling uninfected cells to sites of viral infection (by virtue of chemokine elements displayed on virus particles or displayed on or secreted by infected cells). Exemplary CMV chemokine element genes that can be disabled to produce the viral preparations of the invention include, without limitation, viral genes encoded at the human CMV US27, US28, UL33, UL78, UL146, and UL147 loci, and homologs thereof in non-human CMV. Specific examples of such homologs of HCMV US28 in rhesus monkey include rhCMV US28.1, US28.2, US28.3, US28.4, and US28.5 (SEQ ID NOS. 1-5, respectively; see also FIGS. 1-5B). Additional rhesus homologs are rhUL33 (SEQ ID NO:6; see FIG. 6) and rhUL78 (SEQ ID NO:8; see FIG. 8) (homologs of HCMV UL33 and UL78, respectively), and rhUL33 spliced (SEQ ID NO:7; see FIGS. 7A-B), a splice variant of rhUL33. Additional chemokine element genes in murine CMV for which there are no known homologs in HCMV include mCK-1 and mCK-2.

The second class of viral dissemination genes are genes encoding immune-modulatory proteins, proteins that modulate the anti-viral immune response of an infected host to facilitate viral infection by, for example, (1) causing down-regulation of host MHC Class I (classical) and/or MHC Class II proteins, (2) inhibiting host cell production of cytokines (e.g., stimulatory cytokines IFN-α, IFN-γ, TNF-α, GM-CSF or IL-1α) by cells, e.g., PHA stimulated PBMC and LPS stimulated monocytes, (3) inhibition of cell proliferation (e.g., inhibit Con A/PHA induced proliferation of human and rhesus PBMC), and/or (4) enhancing surface expression of non-classical MHC I on monocytes (modulating protection from natural killer cell activity). Exemplary CMV immune-modulatory genes that can be disabled to produce the viral preparations include, without limitation, viral genes encoded at the HCMV UL111A, US3, US6, US11, US2, UL83, UL18, and UL40 loci, including homologs in other cytomegalovirus strains, e.g., other human CMV clinical strains or non-human strains, such as strains that infect monkeys or mice. As described in greater detail

infra, homologs to each of the foregoing immune-modulatory sequences have been identified by the present inventors in rhesus monkeys with the exception of UL18 and can be utilized in the preparation of the current compositions and in the provided methods.

It will be recognized that the specification of exemplary chemokine element and immune-modulatory genes herein is not intended to be limiting. Other viral chemokine element and immune-modulatory genes, e.g., derived from other clinical strains of CMV or from non-human sources, may differ slightly in sequence or may be identified or discovered in the future. As will be appreciated by those of skill, such genes can be identified by DNA sequencing and cloning (e.g., by amplification using primers based on known sequences).

Various viral genes are described in additional detail in, e.g., §§3.2 and 3.3, and Table 2.

3.2. Viral CMV chemokine elements

As noted, viral chemokine elements refer to viral encoded proteins that are chemokines or chemokine receptors. Viral chemokines are identified by structural similarity (e.g., sequence similarity) to known chemokines (e.g., alpha, beta, gamma or delta chemokines) and biological activities characteristic of chemokines (e.g., chemotactic properties and induction of calcium mobilization activity). Viral chemokine receptors are identified by structural similarity (e.g., sequence similarity) to known chemokine receptors (e.g., seven-transmembrane-domain G protein-coupled receptors) and/or functional tests for chemokine binding, receptor-mediated signal transduction, and imparting chemokine dependent migratory activity on expressing cells. Exemplary viral (CMV) chemokine elements are described below. These viral chemokine elements are homologs of human chemokine receptors involved in cell chemotaxis and targeting.

3.2.1 CMV US28: The CMV genome contains an open reading frame designated US28, which encodes a protein that acts as a functional receptor for certain human and viral chemokines (see, e.g., Gao & Murphy, 1994, *J Biol Chem*. 269:28539-42). The sequence of US28 from HCMV strain VHL/E is found as Genbank accession no. L20501. US 28 sequences from the Toledo strain and AU4.1 strain of HCMV are the same and can be found as Genbank accession no. AF073831. It will be understood by those of skill that other US28 molecules, e.g., derived from other clinical strains of CMV, may differ slightly in sequence (see, e.g., Genbank accession nos. AF 073831-35; see also M.S. Chee, et al., 1990,

Curr. Top. Microbiol. Immunol. 154:125-69), but are also useful in the preparations and methods disclosed herein.

As indicated above, a number of molecules with homology to US28 from HCMV have been identified in rhesus CMV. These homologs are referred to as rhUS28.1, rhUS28.2, rhUS28.3, rhUS28.4 and rhUS28.5. The polynucleotide sequences for these homologs are shown in FIGS. 1-5B and listed as SEQ ID NOS:1-5. There are several factors indicating that these sequences are homologs of human US28. First, the rhUS28 homologs show a relatively high level of similarity with human US28. In particular, there is significant similarity in hydrophobicity/hydrophilicity alignments. The various rhUS28 genes also have hydrophobic and hydrophilic regions consistent with the class of seven member G proteins of which human US28 is a member. Additionally, the rhUS28 family of genes has positional homology with human US28. Given such similarity with human US28, disabling one or more of these genes can be a useful way for attenuating rhCMV preparations provided herein.

Further details regarding the rhUS28 homologs are provided in PCT Application No. 01/27392.

The US28 protein has a high level of sequence similarity to human G protein coupled receptors (Davis-Poynterand Farrell, 1996, *Immunol Cell Biol.* 74:513-22). Upon infection of a cell by CMV, US28 is expressed on the surface of the infected cell and becomes capable of responding to chemokines in the environment. Certain of the inventors have also found that US28 is expressed on virions. US28 has been shown to bind a variety of human, murine, and virus-encoded CC chemokines (Kledal et al., 1997, *Science* 277:1656-9; Kuhn et al., 1995, *Biochem Biophys Res Commun.* 211:325-30). In addition, the CX3C chemokine, fractalkine, binds with a very high affinity (K_I ~50 pM) to US28 (Kledal et al., 1998, *FEBS Lett.* 441:209-14). Fractalkine is expressed on certain endothelial cell surfaces and on populations of dendritic cells (DC), and may thus define a portal through which CMV infected cells or virions go from the circulation to the tissue space, as well as find residence in the DC. US28 has been shown to bind and induce calcium mobilization through RANTES, MCP-1 and MIP1α *in vitro*, induce migration of smooth muscle cells to these ligands following transient or viral expression in vitro, and has been localized to cell and virion membranes.

3.2.2 <u>CMV US27</u>: CMV US27 is a US28 homolog, believed to be the result of a gene duplication event of US28 in HCMV (see, e.g., Chee et al, 1990, *Nature*, 344:774).

3.2.3 CMV UL146 (vCXC1): CMV UL146 encodes a CXC (alpha) chemokine homolog found in human CMV strains. Certain of the inventors have also demonstrated that a UL146 homolog exists in rhesus CMV. vCXC1 shows high inter-strain variability in amino acid sequence, but has conserved structural features, including the ELRCXC motif conserved in all clinical strains sequence to date. The protein encoded by the Toledo strain UL146 has been demonstrated to have neutrophil chemo-attractant properties and acts through the CXCR2 receptor (Penfold et al., 1999, *Proc Natl Acad Sci USA* 96:9839-44). Thus, the vCXC1 polypeptide appears to play a role in neutrophil-mediated dissemination of CMV.

3.2.4 CMV UL147 (vCXC2): UL147 (vCXC2), is a CXC (alpha) chemokine homolog in clinical and low passage strains of HCMV (Cha et al, 1996, *J. Virology* 70: 78-83). Certain of the inventors have discovered that a UL147 homolog is present in rhesus CMV. This ORF shows high inter-strain variability in N terminal amino acid sequence, but has conserved structural features in all clinical strains sequenced to date. The UL147 gene product can induce weak calcium mobilization in THP-1 cells in response to recombinant vCXC2. vCXC2 is expressed as an early protein in infected cells *in vitro*.

3.2.5 CMV UL33: UL33 is a highly conserved G protein coupled receptor and is found in human, mouse and rat CMVs (Davis-Poynter et al., 1997, *J. Virol.* 71:1521-9), and rhesus macaque CMV (see FIG. 6 and SEQ ID NO:6), as well as human herpes virus 6 (Gompels et al., 1995, *Virology* 209:29-51) & human herpes virus 7. The HHV6 homolog (U12) is reported to be a functional receptor for beta chemokines (Isegawa et al., 1998, *J Virol.* 72:6104-12).

Both rat and murine CMV UL33 have been shown to be dispensable for growth in culture but deletion of this ORF leads to decreased virulence in vivo (Beisser et al., 1998, *J Virol.* 72:2352-63; Davis-Poynter, *supra*). Human CMV UL33 has also been localized the envelope of infectious virions (Margulies et al., 1996, *Virology* 225:111-25).

Certain of the inventors have also identified a splice variant of UL33 in rhesus monkeys (see FIGS. 7A and B and SEQ ID NO:7). This particular splice variant can be described with reference to the nucleotide sequence set forth in SEQ ID NO:9 which is a segment that extends roughly 1000 nucleotides upstream of the rhUL33 reading frame and roughly a couple hundred nucleotides downstream. Assigning the first nucleotide of this

sequence as nucleotide 1, with this particular splice variant, translation is initiated at nucleotide 603 through nucleotide 752, exon 1. An intron spanning nucleotide 753 to 830 is removed and exon 1 is joined to exon 2, nucleotide 831 to 2006. In contrast the unspliced gene runs through nucleotide 1017 to 2006 in this sequence. Thus, disabling the foregoing regions involved in the splice variant can also be utilized in the preparation of the compositions herein and in the present methods. Those of skill can identify other such splice variants using programs designed to identify splice variants such as the "Genefinder", "Genehunt" or "GRAIL" programs available at the CMS Molecular Biology resource found at www. unl.edu.

3.2.6 CMV UL78: Like UL33, homologs are present in murine (Rawlinson et al., 1996, *J Virol.* 70:8833-49), rat (Beisser et al., 1999, *J Virol.* 73:7218-30) and rhesus macaque (see FIG. 8 and SEQ ID NO:8) CMVs. Homologs exist in human herpes virus 6 & 7 (Gompels, 1995, *supra*). UL78 appear to encode a G protein coupled receptor, based on the structural characteristics such as trans-membrane spanning regions.

3.2.7 MCK-1, MCK-2: Murine CMV encodes two related CC chemokines (MCK-1 and MCK-2) encoded by alternate splicing of the m131/129 locus. The MCK-1 protein induce Ca^{+2} flux in murine leukocytes *in vitro* (Saederup et al, 1999, *Proc. Nat. Acad. Sci. USA* 96:10881-86). Knockout of the m131/129 locus affects dissemination to the salivary gland, viral clearance from the spleen and liver and aspects of virally induced inflammation in mice *in vivo* (Fleming et al, 1999).

3.3 Viral immune-modulatory genes

As used herein, viral immune-modulatory genes are viral genes that encode proteins that exhibit immune suppressive or other immune modulatory activity, e.g., down-regulation of host MHC molecules, inhibition of host cell production of cytokines and/or inhibition of T cell proliferation. Exemplary viral (CMV) immune inhibitory genes are described below. Although the following information primarily focuses on genes from HCMV, it is anticipated that homologs to these genes exist in other mammalian CMVs, such as rhesus and murine CMV. In fact, certain of the inventors have identified homologs to all of the following genes from HCMV with the exception of UL18. Such sequences can be identified using various existing methods. For example, homologous sequences can be identified by DNA sequencing, cloning (e.g., by amplification using primers based upon

known sequences), hybridization under stringent conditions using known sequences for immune-modulatory genes and by analyzing sequences with sequence comparison algorithms (e.g., BLASTN).

3.3.1 CMV UL111A: The CMV UL111A open reading frame encodes an IL-10-like polypeptide, see, e.g., Lockridge et al., 2000, *Virology* 268:272-80 (Rhesus CMV) and Kotenko et al., 2000, *Proc Natl Acad Sci USA* 97:1695-700 (human CMV). The CMV IL-10 homolog exhibits immune-modulatory activity (e.g., inhibition of production of stimulatory cytokines, inhibition of T lymphocyte proliferation; down-regulation of MHC Class I and II on monocytes. See, e.g., PCT Application No. 01/221831 for additional details concerning rhCMV UL111A.

3.3.2 CMV US3: US3 encodes an endoplasmic reticulum glycoprotein that prevents intracellular transport of MHC Class I molecules, thus inhibiting MHC class I mediated antigen presentation. See U.S. Pat. No. 6,033,671. Murine CMV encodes a homolog protein m152 (Ziegler H et al, Immunity. 1997 Jan;6(1):57-66).

3.3.3 CMV US6: US6 encodes an endoplasmic reticulum glycoprotein that prevents intracellular transport of MHC Class I molecules, thus inhibiting MHC class I mediated antigen presentation. See U.S. Pat. No. 6,033,671.

3.3.4 CMV US11: US11 encodes an endoglycosidase H-sensitive glycoprotein which inhibits surface expression of MHC Class I heavy chains. See U.S. Pat. No. 5,846,806.

3.3.5 US2: The US2 gene product induces export of MHC class I heavy chains from the endoplasmic reticulum via Sec61 (see, e.g., US Pat. No. 6,033,671).

3.3.6 UL83: The US2 gene product inhibits presentation of the 72kDa immediate early antigen to CD8+ T cells (Gilbert et al., 1996, *Nature* 383:720-22).

3.3.7 UL18: UL18 is a viral homolog of the MHC class I molecule which inhibits lysis by natural killer cells by expressing on the infected cell surface (Reyburn et al,

1997, *Nature*, 386: 514-17). Murine CMV encodes a homolog protein m144 (Farrell HE et al, *Nature*. 1997 Apr 3;386(6624):510-4).

3.3.8 UL40: The N terminal fragment of UL40 induces surface expression of HLA-E on infected cells, hence protecting from natural killer cell mediated lysis (Tomasec et al, 2000, *Science*, 287:1031.)

3.3.9 m06: The murine CMV gene product m06 is resident in the endoplasmic reticulum, but upon binding to MHC I molecules is transported to lysosomes, hence facilitating destructruction of bound MHC I molecules Reusch U et al, EMBO J. 1999 Feb 15;18(4):1081-91).

3.3.10 m04: The murine CMV gene product m06 is resident in the endoplasmic reticulum, but upon binding to MHC I molecules is transported to the cell surface. It is believed its interaction with surface MHC I inhibits recognition by T cells or NK cells (Kleijnen et al, EMBO J. 1997 Feb 17;16(4):685-94).

3.3.11 m138: The murine CMV gene product m138 is a homolog of the murine Fc receptor glycoprotein and is able to interfere with humoral immunity to MCMV infected cells (Thale et al, J Virol. 1994 Dec;68(12):7757-65).

TABLE 2

Exemplary Viral Chemokine Elements and Immune-Modulatory Genes

CMV Chemokine Elements or Immune-modulatory Genes	GenBank Accession No.	Reference
US27	X17403	Chee et al, 1990, *Nature*, 344:774
US28	L20501, AF073831-35	Neote et al, 1993, *Cell*, 72:415-25
UL33	X53293	Chee et al, 1990, *Nature*, 344:774
UL78	X17403	Chee et al, 1990, *Nature*, 344:774
UL146	U33331	Cha et al, 1996, *J.Virol*, 70:78-83
UL147	U33331	Cha et al, 1996, *J.Virol*, 70:78-83
UL111A	AF202536	Lockridge et al, 2000, *Virology*, 268:272-80
US11	X17403	U.S. pat. no. 5,846,806
US3	X17403	U.S. pat. no. 6,033,671
US6	X17403	U.S. pat. no. 6,033,671
US2	X17403	U.S. pat. no. 6,033,671
UL83	X17403	Gilbert et al. 1996 *Nature* 383, 720-722
UL18	X17403	Reyburn et al, 1997 *Nature*, 386: 514-517
UL40	X17403	Tomasec et al, 2000, *Science*, 287:1031

3.4 Disabling CMV Dissemination Genes

Viral dissemination genes can be disabled in any of a variety of ways known in the art to produce the preparations of the invention, including: insertional mutagenesis in the promoter or protein-coding region, or partial or complete deletion of the target gene. In one embodiment, as described *infra*, the CMV target gene is disabled by insertion of a heterologous sequence encoding a human chemokine element. In designing primers, probes, and cloning strategy, useful CMV gene sequences are found in the scientific literature, including GenBank, or can be determined by routine methods based on known sequences. Methods for deletion and other forms of mutagenesis are well known, see, e.g., Ausubel at Chapter 8.

4. Heterologous Chemokine Elements

4.1 Generally

Certain of the modified CMV provided herein have incorporated in the CMV genome a heterologous nucleotide sequence encoding a heterologous (e.g., exogenous) chemokine element. As noted *supra*, the term "chemokine element" refers to chemokines, chemokine receptors and chemokine binding proteins. Without intending to be limited by

any mechanism, the expression of the inserted heterologous chemokine element, as discussed *infra*, results in motility of the cells infected with the modified virus and particularly, dissemination of the cells to host immune cells and tissues. Preferred heterologous chemokine elements are those that mediate migration of cells in which they are normally expressed to immune system cells and tissues (e.g., secondary lymphoid organs) or, conversely, mediate migration of immune system cells (e.g., APCs) toward the cells or tissues that normally express the chemokine element. Useful heterologous chemokine elements include those identified as chemoattractant immune system cells (e.g., dendritic cells, or immature dendritic cells but not chemoattractant for one or more of mature dendritic cells, neutrophils, T-lymphocytes, monocytes, eosinophils). Such elements include those described in, or identified using the methods described in PCT Publication WO 01/80887.

Exemplary chemokine elements used in the practice of the invention are described, *infra* (§§4.1.1-4.1.5, and Table 3). Additional chemokine elements that may be used are known, see, e.g., the R&D Systems Catalog (1999) and (2000) R&D Systems Inc., 614 McKinley Place N.E. MN 55413, the R&D online catalog at www.rndsystems.com (e.g., January 1, 2001), the CYTOKINE FACTS BOOK, 1994, Academic Press Ltd., the CHEMOKINE FACTS BOOK, 1997, Academic Press Ltd., all incorporated by reference, and the GenBank protein sequence database http://www.ncbi. nlm.nih.gov/entrez/query.fcgi).

Typically, when the compositions of the invention are used for inducing an immune response in an animal, the chemokine element is selected to be from the same species as the host contemplated for the vaccine. For example, for a vaccine intended for use in human subjects, a human chemokine element is inserted into a virus able to infect human cells. Similarly, if a vaccine is intended for use in Rhesus monkeys, then a Rhesus chemokine element is inserted into a virus able to infect Rhesus cells. However, certain chemokine elements have cross-species activity and so it is not a requirement that the chemokine be from the same species as the host. While the following sections discuss primarily human chemokine elements, corresponding homologs are expected to be found in other mammals (e.g., monkeys and mice). As described supra in § 3.2, such homologs can be identified by sequence similarity and biological activity similarity to known chemokine elements (e.g., chemokines or chemokine receptors).

Exemplary chemokine elements include, without limitation, the following:

4.1.1 <u>CC chemokine receptor-7 (CCR7)</u>: CCR7 (EBI1) is a CC chemokine receptor belonging to a subfamily of G protein coupled receptors. CCR7 is most closely

related to CCR9 and CCR6. CCR7 is expressed on mature dendritic cells, activated B cells, naïve T cells, as well as central memory T cells (Jourdan et al., 2000, *J Immunol.* 165:716-24; Sozzani et al., 1998, *J Immunol.* 161:1083-6; Yanagihara et al.,1998, *J Immunol.* 161:3096-102; Yoshida, et-al., 1998, *Int Immunol.* 10:901-10). CCR7 and its ligands are key regulators in cellular trafficking to the lymph nodes and other secondary lymphoid organs (SLO).

CCR7 responds to two CC chemokines: SLC (also called (CCL21, 6Ckine, TCA4, and Exodus 2) and ELC (also CCL19, MIP3 beta, EB 1 ligand, and CKb11). Experiments in CCR7 knockout mice have indicated that CCR7 plays an important role in organizing the primary immune response (Forster et al., 1999, *Cell* 99:23-33). Both CCR7 and its ligands play a significant role organizing the events which are required to generate an efficient primary immune response especially by facilitating co-localization of antigen presenting cells and immune effector cells by inducing their migration to secondary lymph organs (SLOs). Expression of CCR7 on cells infected with engineered virus results in homing of these cells to SLO where CCR7 ligands, ELC and SLC are expressed, i.e., specifically in areas where antigen presentation and immune education take place. Alternatively CCR7 on membrane of viral particles can lead free virus in the lymph to adhere to these regions through GAG bound ELC/SLC and hence be able to initiate an immune response.

The role of CCR7 and its ligands in cellular trafficking to the lymph nodes and other secondary lymphoid organs is described in, for example, Iwasaki et al., 2000, *J Exp Med* 191:1381-94; Forster et al., 1999, *Cell* 99:23-33; Sallusto et al., 1999, *Eur J Immunol* 29:1617-25; Kellermann et al., 1999, *J Immunol* 162:3859-64; Saeki et al., 1999, 162:2472-5; Dieu et al., 1998, *J Exp Med* 188:373-86; Gunn et al., 1999, *J Exp Med* 189:451-60.

4.1.2 <u>ELC/SLC</u>: ELC and SLC are CCR7 ligands. These chemokines are capable of inducing a calcium response in CCR7-expressing cells, and cells expressing CCR7 will migrate to a gradient of ELC or SLC (Yoshida et al., 1997, *J. Biol Chem.* 272:13803-9; Yoshida et al., 1998, *J Biol Chem.* 273:7118-22. Both SLC and ELC are constitutively expressed in secondary lymph organs. ELC and SLC have also been shown to bind only one other receptor originally and provisionally identified as CCR11 (Gosling et al., 2000, *J. Immunol.* 164:2851-6).

4.1.3 MIP3α: MIP3α (also known as LARC, Exodus, or CCL20) is the ligand for CCR6, forming an exclusive receptor/ligand pair (Baba et al., 1997, *J Biol Chem.* 272:14893-8). Immature dendritic cells expressing CCR6 can also be generated *in vitro* from monocytes stimulated with TGF-beta 1 (Yang et al., 1999, *J Immunol.* 163:1737-41). Expression of MIP3α at specific anatomical sites is believed to induce influx of CCR6 bearing immature dendritic cells, hence enhancing localization and priming of these key antigen presenting cells (Dieu et al., 1998, *J Exp Med.* 188:373-86). Thus, expression of MIP3 α by infected cells, either secreted or surface bound, is believed to cause targeting by immature dendritic cells which express CCR6. Without intending to be bound by a particular mechanism, virally infected cells expressing MIP3α attract APC, which take up virus-encoded antigen, mature and then migrate to SLO to stimulate immunity. CCR6, the only known receptor for MIP3α, is expressed *in vivo* predominately on the surface of lymphocytes and resident Langerhan cells (i.e., skin dendritic) (Greaves et al., 1997, *J Exp Med.* 186:837-44). A GenBank Accession no. is Xm 004279.

4.1.4. MIP1β: Human MIP1beta (accession number J04130, Lipes et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:9704) is a CC chemokine and has a murine homologue (Graham et al., 1990, *Nature* Vol. 344:442). It has highly selective chemoattractive activity to monocyte derived immature dendritic cells (see, e.g., PCT Application No. 01/12162, filed April 12, 2001).

4.1.5. MC10: Mouse C10 is a CC chemokine (Accession number AF293362) with no known human counterpart (Orlofsky et al, 1991, *Cell Regulation*, 2:403-12) and is expressed primarily from mouse macrophage cells. It has homology to the MIP1 family (Orlofsky, A et al, *Cell Regulation*, Vol 2, p403-412, 1991). Highly selective *in vitro* migratory activity on human monocyte-derived immature dendritic cells has been demonstrated (see, e.g., PCT Application No. 01/12162, filed April 12, 2001).

4.1.6 mMDC: (Accession number AF076596) is a member of the murine CC chemokine family produced by activated B lymphocytes and dendritic cells (Schaniel et al, 1998, *J. Exp. Med.*, 188:451-63) and has a human homologue called MDC (Godiska et al, *J. Exp. Med.* 185:1595-1604). The human version has been reported to have activity towards monocytes, monocyte-derived dendritic cells, and natural killer cells (Godiska, *supra*). Certain of the inventors have shown, however, that in *in vitro* assays, murine MDC has

selective activity towards human monocyte-derived immature dendritic cells (see, e.g., PCT Publication WO 01/80887.

TABLE 3
Exemplary Non-Viral Chemokine Elements

Chemokine element	GenBank Accession No.
CCR7	XM 008651
MIP3α	XM 002224
ELC	XM 005637
SLC	XM 005633
MIP1β	J04130
C10	AF293362
MDC	AF076596

4.2 Introduction of heterologous chemokine elements into virus

With certain modified CMV compositions which are provided, a heterologous chemokine element (e.g., human chemokine or chemokine receptor gene) is introduced into the virus genome along with regulatory elements that permit expression of the chemokine element in infected cells (i.e., on the surface of infected cells, secreted by infected cells, and/or on the surface of virions produced in infected cells). This can be accomplished in a variety of ways routine in the molecular biology arts, including, for example, by using an expression cassette. To express the gene product of interest, the coding sequence is operably linked to a promoter sequence. In addition, when the encoded protein is to be secreted by the cell, a secretion leader sequence is included. The secretion leader sequence may be naturally associated with the introduced gene (e.g. for genes encoding naturally secreted products) or may by synthetic or heterologous (e.g., inserted into the DNA sequence at the protein or peptide amino-terminus to encode a fusion protein). See, e.g., Ausubel, *supra*, and Sambrook, *supra*.

Suitable promoters are typically constitutive, but may be constitutive, cell type-specific, stage-specific, and/or modulatable or regulatable (e.g., by hormones such as glucocorticoids). Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPSV promoter, the tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), the constitutive CMV promoter, and promoter-enhancer combinations known in the art. As noted *supra*, other regulatory elements may also be required or desired

for efficient expression of a polynucleotide and/or translation of a sequence encoding proteins. For translation, these elements typically include an ATG initiation codon and adjacent ribosome binding site or other sequences. For sequences encoding full-length proteins or fragments including the initiation codon, no additional translational signals may be needed. However, in cases where only a protein fragment is inserted, exogenous transcriptional and/or translational control signals (e.g., the promoter, ribosome-binding site, and ATG initiation codon) must often be provided. In addition, the efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the host or cell system in use. For example, the SV40 enhancer or CMV enhancer may be used to increase expression. Additionally, a poly A sequence can also be included.

As noted *supra*, in some embodiments targeting substitutions of endogenous viral chemokine and chemokine receptor genes are replaced with exogenous (e.g., human) chemokine and chemokine receptor genes, for example: US28 → CCR7; vCXC1 (UL146) and/or vCXC2 (UL147) → ELC and/or SLC ("→" = replaced with).

5. Immunogen Expression

5.1 Choice of Immunogen

Exemplary pathogens (or agents) and genes encoding antigens are provided in Table 4 and additional examples of immunogens are described *infra*. According to the invention, the immunogenic polypeptide is characteristic of a pathogenic organism (e.g., virus, bacterium, fungus, parasite or protozoan) or is a tumor antigen. Candidate immunogens are those for which it is known, believed, expected or proposed that, if elicited, an immune response to the immunogen in an animal would be therapeutic or protective from disease. Thus, antigens not associated with pathogenic organisms, such as reporter genes (e.g., SEAP, GFP; β-glucuronidase; His6 reporter tag), antibiotic resistance genes used in genetic engineering (e.g., resistance to chloramphenicol, kanamycin, G418, bleomycin and hygromycin), and the like are usually not suitable as immunogens for the present compositions.

It will be appreciated that it is not necessary (or always desirable) to express the entire polypeptide encoded by a pathogen, and typically, one or more an immunogenic fragments of the polypeptides are expressed. When fragments are used, suitable immunogenic sequences are known or can be determined using routine art-known methods. See, e.g., Ausubel chapter 11.15. Typically, the expressed immunogenic polypeptide is at least 6 amino acids in length, more often at least about 8, and sometimes at least about 10, at

least about 20, at least about 50 residues, or even full-length. In some instances, the immunogenic polypeptide is expressed as a fusion polypeptide.

TABLE 4
Exemplary Antigens

Pathogen/ Biothreat Agent	**Gene**	**GenBank Accession No.**
Bacillus anthracis	PA (protective antigen)	M22589
Dengue	NS1	M58486
Yersinia pestis	F1	X61996
Ebola	GP NP	U31033 J04337
Marburg	GP NP	X68493 X68495
Lassa	N GPc	K03362 J04324
Venezulean equine encephalitis virus (VEE)	GP	L04598
Eastern equine encephalitis virus (EEE)	GP	D00145

Additional exemplary antigens or vaccine components include antigens derived from microbial pathogens such as bacteria (e.g., Pertussis, Cholera, Meningitis, Borrelia burgdorferi, Haemophilus B, Streptococcs pneumoniae, Typhoid), and viruses (e.g., in addition to Herpes viruses, e.g., Influenza virus; Hepatitis A; Hepatitis B; Measles; Rubella virus; Mumps, Rabies; Poliovirus; Japanese Encephalitis virus; Rotavirus; Varicella; and Smallpox), Diphtheria (Corynebacterium diphtheriae) and Tetanus (Clostridium tetani). Other antigens are derived from parasites. In an embodiment, the antigen is a tumor associated antigen (tumor specific antigen) and the reagents of the invention are used to treat cancers, including, but not limited to, melanomas, lung cancers, thyroid carcinomas, breast cancers, renal cell carcinomas, squamous cell carcinomas, brain tumors and skin cancers.

Expression of a desired sequence can be accomplished using routine methods for, e.g., expressing a selected sequence in a mammalian cell. Promoters such as those described *supra*, §4.3, can be used to express an immunogenic protein. Multicistronic constructs or fusion protein-encoding sequences can also be used in the construction of the recombinant viruses.

6. Administration of Immunogenic Compositions

6.1 CMV Strains

Typically, the CMV strain that is modified for use in the methods provided herein is matched to the intended host. For example, a vaccine intended for use in human subjects uses a human CMV (modified as described herein), such as CMV AD169, CMV Towne, CMV Davis, CMV Toledo. Similarly, for a vaccine intended for use in Rhesus monkeys, a monkey CMV (modified as described herein), such as CMV Rh68.1, CMV CSG is used. In any event, the virus used in formulation of a vaccine should be able to infect at least some cells of the host organism.

6.2 Formulation and administration

When used for vaccination, vaccination with the compositions of the invention may be prophylactic vaccination (wherein the vaccine is administered prior to exposure, or anticipated exposure, to the target antigen, e.g., to a subject susceptible to or otherwise at risk of exposure to a disease) and/or immunotherapeutic vaccination (wherein the vaccine is administered after exposure to the target antigen to accelerate or enhance the immune response).

The vaccine preparations of the invention are typically administered intradermally or subcutaneously, but can also be administered in a variety of ways, including orally, by injection (e.g., intradermal, subcutaneous, intramuscular, intraperitoneal and the like), by inhalation, by topical administration, by suppository, using a transdermal patch.

When administration is by injection, the compositions may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

In some instances, multiple preparations of recombinant viruses (e.g., each encoding and expressing a different antigen or immunogenic polypeptide) can be administered.

Typically, an amount of the viral composition will be administered to the subject that is sufficient to immunize an animal against an antigen (i.e., an "immunologically effective dose" or a "therapeutically effective dose"). The effective dose can be formulated in animal models to achieve an induction of an immune response using techniques that are well known in the art. Exemplary doses are 10 to 10^7 pfu per dose, e.g., 10 to 10^6 or 10^3 to

10^6 pfu. One having ordinary skill in the art can readily optimize administration to humans (e.g., based on animal data and clinical studies).

In various embodiments, the compositions include carriers and excipients (including but not limited to buffers, carbohydrates, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents, suspending agents, thickening agents and/or preservatives), other pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, and/or a conventional adjuvant (e.g., Freund's Incomplete Adjuvant, Freund's Complete Adjuvant, Merck Adjuvant 65, AS-2, alum, aluminum phosphate, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). It will be recognized that, while any suitable carrier known to those of ordinary skill in the art may be employed to administer the compositions, the type of carrier will vary depending on the mode of administration. Compounds may also be encapsulated within liposomes using well known technology.

The compositions are usually produced under sterile conditions and may be substantially isotonic for administration to hosts. Typically, the compositions are formulated as sterile, and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

7. Examples

7.1 General Virology Methods

General methods handling CMV, and for constructing CMV genomic mutants are known to practitioners of the virology art. See, e.g., Mocarski et al., 1996, *Intervirology* 39:320-30, 1996; Spaete et al, 1987, *Proc. Natl. Acad. Sci. USA* 84:7213-17; Ehsani et al, 2000, *J. Virol*, 74:8972-79.

7.1.1 Viral DNA Preparation

Viral DNA can be prepared from infected cells using the method of Ehsani et al., 2000, *J. Virol* 74:8972-9, or other suitable methods.

7.1.2 Propagation of Virus and Cells

Virus and cells are propagated by standard methods. Human CMV is propagated in human fibroblasts, e.g., human dermal fibroblast (HDF, Clonetics, CA),

MRC-5 (ATCC # CCL 171) and others, grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum supplemented with amino acids and antibiotics according to standard techniques (Spaete and Mocarski, 1985, *J. Virology* 56:135-43). Murine CMV is propagated in mouse fibroblasts, e.g. NIH/3T3 embryonic mouse fibroblasts (ATCC No. CRL-1658) and others, grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum supplemented with amino acids and antibiotics according to standard techniques. Rhesus CMV is propagated in rhesus fibroblasts, e.g. DBS-FRhL-2 rhesus lung fibroblasts (ATCC No. CL-160) and others, grown in DMEM with 10% fetal bovine serum supplemented with amino acids and antibiotics as per standard techniques.

7.2 EXAMPLE 1

Simultaneous Knock-Out of Viral Gene and Insertion of Heterologous Chemokine Element into a CMV Genome.

This example describes a general protocol for the construction of the recombinant cytomegaloviruses described in the application. The recombinants may include 1) disabled viral chemokine element or immune-modulatory genes 2) insertion of a heterologous gene encoding a chemokine element 3) insertion of a foreign antigen gene or 4) combinations of the above.

In one embodiment the viral (e.g., CMV) gene to be disabled is replaced with a gene encoding a heterologous chemokine element, a heterologous antigen gene and/or a selectable marker by constructing a targeting plasmid vector. The targeting vector contains the heterologous chemokine element, a heterologous antigen gene and/or a selectable marker operably linked to regulatory sequences and flanked by sequence from the viral gene (typically at least 300, and often at least 500, base pairs of viral sequence is present at each end of the insert, i.e., the 5' and 3' ends). In constructing the targeting vector, one suitable approach is to clone the viral gene into a (plasmid) vector and insert an expression cassette encoding the entire heterologous chemokine element, a heterologous antigen gene and/or a selectable marker with or without the removal of CMV sequence. It will be apparent to one of ordinary skill that these constructs can be prepared using routine molecular biological techniques, e.g., insertion of synthetic, amplified or subcloned gene fragments of interest into existing restriction sites in a target sequence; introduction of additional restriction sites using synthetic linkers, site-directed mutagenesis; and the like.

Regulatory sequences typically include a promoter active in cells of the host (e.g. the CMV major immediate early promoter, CMV MIEP), an internal ribosome entry site (IRES) (e.g. the EMC IRES), a selectable marker (e.g. the puromycin resistance gene, green fluorescent protein, etc.), and a polyadenylation signal (e.g. the SV40 polyadenylation signal).

In an alternate approach sequence encoding the heterologous chemokine element, a heterologous antigen gene and/or a selectable marker may be inserted into an inter-genic space with or without the removal of CMV sequence. Appropriate loci may include but are not limited to the inter-genic space between CMV US1 and US2, UL108 and UL109 and other loci. Sequence is inserted by construction of a targeting vector. The targeting vector contains the heterologous chemokine element, a heterologous antigen gene and/or a selectable marker operably linked to regulatory sequences and flanked by sequence from the viral gene (typically at least 300, and often at least 500, base pairs of viral sequence is present at each end of the insert, i.e., the 5' and 3' ends). In constructing the targeting vector, one suitable approach is to clone the viral loci into a (plasmid) vector and insert an expression cassette entire encoding the heterologous chemokine element, a heterologous antigen gene and/or a selectable marker with or without the removal of CMV sequence. It will be apparent to one of ordinary skill that these constructs can be prepared using routine molecular biological techniques, e.g., insertion of synthetic, amplified or subcloned gene fragments of interest into existing restriction sites in a target sequence; introduction of additional restriction sites using synthetic linkers, site-directed mutagenesis; and the like.

Regulatory sequences typically include a promoter active in cells of the host (e.g. the CMV major immediate early promoter, CMV MIEP), an internal ribosome entry site (IRES) (e.g. the EMC IRES), a selectable marker (e.g. the puromycin resistance gene, green fluorescent protein, etc.), and a polyadenylation signal (e.g. the SV40 polyadenylation signal).

In an alternate strategy the foreign gene may be inserted into a recombinant genome at the same locus as a previous insertion. For instance the heterologous chemokine element, a foreign antigen gene and/or a selectable marker may be targeted to replace the selectable marker used in the previous insertion of another heterologous gene (e.g., a chemokine element). Sequence is inserted by construction of a targeting vector. The targeting vector contains the heterologous chemokine element, a heterologous antigen gene and/or a second selectable marker operably linked to regulatory sequences and flanked by

sequence from the first selectable marker or regulatory element (typically at least 300, and often at least 500, base pairs of viral sequence is present at each end of the insert, i.e., the 5' and 3' ends). In constructing the targeting vector, one suitable approach is to clone sequence from the first selectable marker into a (plasmid) vector and insert an expression cassette encoding the heterologous chemokine element, a heterologous antigen gene and/or a selectable marker with or without the removal of selectable marker sequence. It will be apparent to one of ordinary skill that these constructs can be prepared using routine molecular biological techniques, e.g., insertion of synthetic, amplified or subcloned gene fragments of interest into existing restriction sites in a target sequence; introduction of additional restriction sites using synthetic linkers, site-directed mutagenesis; and the like.

CMV recombinants are generated by co-transfection of the targeting vector and wild type CMV viral DNA or recombinant CMV viral DNA into dermal fibroblasts of the appropriate species (e.g., human dermal fibroblasts, rhesus dermal fibroblasts, murine 3T3 fibroblasts) using calcium phosphate transfection. Cells containing recombinant virus are purified from wild type infected cells by selection with antibiotic, colormetric selection, or the like. Selected infected cells are then subjected to plaque purification by standard techniques to obtain a pure recombinant virus preparation.

The purified virus preparation is then combined with a suitable carrier (e.g., physiological saline containing a stabilizer) for administration to a host animal.

7.3 <u>EXAMPLE 2</u>

<u>Simultaneous Knock-Out of Human CMV US28 Gene and Insertion of Human CCR7 Coding Sequence</u>

This example describes production of human CMV recombinants in which US28 has been disabled and human CCR7 coding sequences operably linked to a promoter have been inserted.

Using viral DNA extracted from human CMV strain AD169 (Genbank Accession no. x17403) virions as template, PCR amplification is carried out using the primers: gtgaattcggttggttccccgtgttt (AD27up) and gcggatcctcgcgagtcgcgtcttcatcgtag (AD28low) to amplify AD27/28. The resulting 822 bp fragment is purified, digested with *Bam*HI and *Eco*RI, and cloned into *Bam*HI and *Eco*RI digested vector (pGEM3 (Promega)). The resulting construct is called pGEM28.1.

Using the same viral DNA template as above, PCR amplification is carried out using the primers: gtggatcctcgagcgctcgccttttgtcact (AD28up) and gcggatcccccccgccc

accatacaac (AD29low) to amplify AD27/28. The resulting 877 bp fragment is purified, digested with *Bam*HI, cloned into pGEM28.1, cut with *Bam*HI with the end homologous to AD28up juxtaposed to sequence homologous to AD28low. The resulting construct is called pGEM28.2

To clone the human CCR7 receptor (CCR7 mRNA sequence, Genbank Accession no. XM 049959), whole cell RNA is isolated from human PBMC (e.g. using commercially available kits, Qiagen, CA). PCR amplification is carried out using the primers: gcgaattcagcgtcatggacctgggg (ccr7up) and tggaattcagaagagtcgcctatggg (ccr7low) to amplify CCR7.1. The resulting 1172 bp product is purified, digested with *Eco*RI, and cloned into pIRESpuro (Clontech) at the *EcoR*I site. The resulting construct is called pIRES CCR7.1

pIRES CCR7.1 is digested with *Nru*I and *Xho*I. The 3861 bp restriction fragment contains (5' to 3') the CMV major immediate early promoter (CMV MIEP) CCR7 coding sequence, the EMC internal ribosome entry site (IRES), the puromycin resistance gene, and the SV40 polyadenylation signal. This fragment is cloned into pGEM28.2 digested with *Nru*I and *Xho*I. The resulting construct is called pGEM28 CCR7. These steps remove 771 bp of CMV US28, and insert a 3861 bp fragment containing CMV MIEP/CCR7/IRES/puro/polyA (i.e., 3090 bp total insertion).

CMV recombinants are generated by co-transfection of this vector (pGEM28 CCR7) and wild type CMV viral DNA into human dermal fibroblasts (Clonetics) using calcium phosphate transfection. Cells containing recombinant virus are purified from wild type infected cells by selection with the antibiotic puromycin. Selected infected cells are then subjected to plaque purification by standard techniques to attain a pure recombinant virus preparation.

7.4 EXAMPLE 3

Simultaneous Knock-Out of Murine CMV UL78 Gene and Insertion of Murine CCR7 Coding Sequence

This example describes production of murine CMV recombinants in which UL78 has been disabled and murine CCR7 coding sequences operably linked to a promoter and selectable marker have been inserted.

Using viral DNA extracted from murine CMV (e.g., strain Smith (Genbank Accession no. U68299)) virions as template, PCR amplification is carried out using the primers: ataagaatgcggccgctcgactacatgctgctgc (S78.1) and cggaattccgtccggctgctgcgcttcttc (S78.2). The 2351bp PCR fragment is isolated and digested with *Not*I and *Eco*RI, and cloned

into *Not*I and *Eco*RI digested vector (pGEM11 (Promega)). The resulting construct is called pGEMm78.

To clone the murine CCR7 receptor (mCCR7 mRNA sequence, Genbank Accession no. NM_007719), murine genomic DNA is isolated from murine PBMC (e.g. using commercially available kits, Qiagen, CA). PCR amplification is carried out using the primers: ataagaatgcggccgctgacccagggaaacccagg (mCCR7up) and cggaattccgtcagctcctgggagaggtccttg (mCCR7low) to amplify mCCR7. This fragment is digested with *Not*I and *Eco*RI and cloned into *Not*I and *Eco*RI digested pIRESpuro (Clontech) to give pIRESpuromCCR7. The pIRESpuromCCR7 vector construct is digested with *Nru*I and *Xho*I, generating a DNA fragment which encodes the CMV major immediate early promoter (CMV MIEP), mCCR7 coding sequence, the EMC internal ribosome entry site (IRES), the puromycin resistance gene, and the SV40 polyadenylation signal. The purified *Nru*I-*Xho*I fragment is then cloned into the pGEMm78 vector digested with *Sma*I and *Xho*I. These sites are compatible with the *Nru*I and *Xho*I sites of the mCCR7 fragment for ligation. The resulting construct (pGEMm78IRESmCCR7) contains the m78 gene disrupted by the insertion of the mCCR7 gene and an IRES driven puromycin selection marker.

CMV recombinants are generated by co-transfection of this vector (pGEMm78IRESmCCR7) and wild type murine CMV (e.g., strain Smith) viral DNA into murine 3T3 fibroblasts (ATCC # CCL92) using calcium phosphate transfection. Cells containing recombinant virus are purified from wild type infected cells by selection with the antibiotic puromycin. Selected infected cells are then subjected to plaque purification by standard techniques to attain a pure recombinant virus preparation.

7.5 EXAMPLE 4

Insertion of Bacillus anthracis protective antigen gene into US28/CCR7 recombinant CMV.

This example describes insertion of a Bacillus anthracis protective antigen gene into the genome of the US28/CCR7 recombinant CMV described in Example 2.

Using viral DNA extracted from human CMV strain AD169 (Genbank Accession no. x17403) virions as template, PCR amplification is carried out using the primers: gcggtaccgcgacgccgtcgctggg (108 up) and tggatccgtcagggaaatacaag (108 low) to amplify AD108. The resulting 1300 bp fragment is purified, digested with *Bam*HI and

KpnI, and cloned into *Bam*HI and *Kpn*I digested vector (pGEM3 (Promega)). The resulting construct is called pGEM108.

Using the same viral DNA template as above, PCR amplification is carried out using the primers: atggatcctcttctatcacggtggc (109 up) and gcggatccaggatcgatttcgtgcg (109 low) to amplify AD109. The resulting 1085 bp fragment is purified, digested with *Bam*HI, and cloned into pGEM108 cut with *Bam*HI with the end homologous to AD109up juxtaposed to sequence homologous to AD108low. The resulting construct is called pGEM108/109.

To clone the Bacillus anthracis protective antigen (BAPA; Genbank Accession no. M22589), DNA is isolated from bacilli containing the BAPA sequence (e.g., pBLSCRPPA from Iacono-Connors L.C. at U.S. Army Medical Research Institute of Infectious Diseases, Frederick MA) by routine means (e.g. using commercially available kits from Qiagen, CA) and used as template for PCR with the primers ggcccggggaagttaaacaggagaaccg (BAPAup) and gggatatcttaccttatcctatctcat (BAPAlow). The resulting 2229 bp product is purified, cut with *Eco*RV and *Xma*I, and cloned into *Eco*RV and *Xma*I-digested vector (Clontech pIREShyg2). The resulting construct is called pBAPAiresHyg.

The following complimentary oligo sequences containing the Ig kappa leader sequence (Acc# D84070) are synthesized:

5'-ctagcatggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtgaccc-3'

5'-ccgggggtcaccagtggaacctggaacccagagcagcagtacccatagcaggagtgtgtctgtctccatg-3'

and annealed to give a double-stranded oligonucleotide with overhangs for the restriction enzymes NheI and a XmaI. This synthetic sequence is cloned into *Nhe*I and *Xma*I-digested pBAPAiresHyg. The resulting construct is called pSecBAPAiresHyg.

pSecBAPAiresHyg is digested with *Xho*I then subjected to a partial digest with *NruI*. The 5560 bp restriction fragment contains (5' to 3') the CMV major immediate early promoter (CMV MIEP), the Ig kappa leader sequence, the BAPA coding sequence, the EMC internal ribosome entry site (IRES), the hygromycin resistance gene, and the SV40 polyadenylation signal. This fragment is cloned into pGEM108/109 digested with *Nru*I and *Xho*I. The resulting construct is called pGEM108/109 BAPA. These steps remove 1058 bp of CMV inter-genic sequence between the AD169 UL108 and UL109 ORFs, and insert a 5560 bp fragment containing CMV MIEP/secreted BAPA/IRES/hygro/polyA (i.e., 4502 bp total insertion).

Viral DNA is purified from virions following infection of human dermal fibroblasts with a recombinant CMV strain prepared as described in Examples 1-3. CMV double recombinants are generated by co-transfection of this vector (pGEM108/109 BAPA) and the recombinant CMV viral DNA into human dermal fibroblasts (Clonetics) by calcium phosphate precipitation. Cells containing double recombinant virus are purified from wild type infected cells by selection with an antibiotic (e.g. hygromycin). Selected infected cells are then subjected to plaque purification by standard techniques to attain a pure double recombinant virus preparation.

7.6 EXAMPLE 5

Simultaneous Knock-Out of Rhesus CMV UL33 Gene and Insertion of a Foreign Antigen Gene

This example describes production of rhesus CMV recombinants in which rhesus UL33 has been disabled and rhesus CCR7 coding sequences operably linked to a promoter have been inserted.

Using viral DNA extracted from rhesus CMV (e.g., strain Rh68.1 (ATCC # VR 677)), virions as template, PCR amplification is carried out using the primers: cggaattcctctttagtcggcagggtctt (Rh32up) and ctggatccgtggctttgtctttggctttt (Rh33low) to amplify Rh32/33. The resulting 1404 bp fragment is purified, digested with *Bam*HI and *Eco*RI, and cloned into *Bam*HI and *Eco*RI digested vector (pGEM3 (Promega)). The resulting construct is called pGEM32-33.

To clone the Bacillus anthracis protective antigen (BAPA; Genbank Accession no. M22589), DNA is isolated from bacilli containing the BAPA sequence or from a plasmid containing this sequence (e.g., pBLSCRPPA from Iacono-Connors L.C. at U.S. Army Medical Research Institute of Infectious Diseases, Frederick MA) by routine means (e.g. using commercially available kits from Qiagen, CA) and used as template for PCR with the primers ggcccggggaagttaaacaggagaaccg (BAPAup) and gggatatcttaccttatcctatctcat (BAPAlow). The resulting 2229 bp product is purified, cut with *Eco*RV and *Xma*I, and cloned into *Eco*RV and *Xma*I-digested vector (Clontech pIREShyg2). The resulting construct is called pBAPAiresHyg.

The following complimentary oligo sequences containing the Ig kappa leader sequence (Acc# D84070) are synthesized:

5'-ctagcatggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtgaccc-3'

5'-ccgggggtcaccagtggaacctggaacccagagcagcagtacccatagcaggagtgtgtctgtctccatg-3' and annealed to give a double-stranded oligonucleotide with overhangs for the restriction enzymes *Nhe*I and a *Xma*I. This synthetic sequence is cloned into *Nhe*I and *Xma*I-digested pBAPAiresHyg. The resulting construct is called pSecBAPAiresHyg.

pSecBAPAiresHyg is digested with *Xho*I then subjected to a partial digest with *NruI*. The 5560 bp restriction fragment contains (5' to 3') the CMV major immediate early promoter (CMV MIEP), the Ig kappa leader sequence, the BAPA coding sequence, the EMC internal ribosome entry site (IRES), the hygromycin resistance gene, and the SV40 polyadenylation signal. This fragment is blunted using Deep Vent or Klenow enzyme (New England Biolabs) to give a 5560 bp fragment containing CMV MIEP/secreted BAPA/IRES/hygro/polyA and cloned into pGEM32-33 digested with *MscI* and *PshA*I so as to remove the central portion of the rhesus UL33 ORF.

The resulting construct is called pGEM33 BAPA. These steps remove 852 bp of rhesus CMV UL33, and insert a 5560 bp fragment containing CMV MIEP/secreted BAPA/IRES/hygro/polyA (i.e., 4708 bp total insertion).

CMV recombinants are generated by co-transfection of this vector (pGEM33 BAPA) and wild type or recombinant rhesus CMV viral DNA into rhesus dermal fibroblasts using calcium phosphate transfection. Cells containing recombinant virus are purified from wild type infected cells by selection with an appropriate antibiotic (e.g., hygromycin). Selected infected cells are then subjected to plaque purification by standard techniques to attain a pure recombinant virus preparation.

7.7 EXAMPLE 6

Alternative Virus Construction Strategy

This example describes an alternative cloning strategy for viral recombinants. CMV (e.g., Rhesus) viral recombinants are constructed with modifications of UL111A and US28, and other genes using a yeast shuttle vector (e.g., see Larionov et al., 1996, *Proc Natl Acad Sci USA* 93:491-6). Rhesus CMV sequence (hooks) is inserted by standard bacterial cloning techniques, into CEN6$^+$ yeast shuttle vector pVC604 (recombinants are not viable in yeast as deficient in the yeast autonomous replicating sequence (ARS)). A pGEM vector containing sequence homologous to the gene of interest is constructed with early termination signals, a FLAG epitope tag and a yeast ARS incorporated in the ORF by PCR based mutagenesis. Spheroplast transformation of recombinagenic yeast strain VL6-48 with above vectors and viral genomic DNA using highly efficient TAR cloning methodologies

(transformation associated recombination (Larionov et al, 1996, *Proc Natl Acad Sci USA* 93:491-6). Only triple recombination events are viable in yeast due to ARS/CEN6 selection. Retrofitting of yeast vector, using TAR, with a bacterial F factor origin of replication (with plasmid BRV-1 and His selection) allowing carriage and amplification of the vector in bacteria (chloramphenicol selection). Anion exchange isolation of vector DNA from bacteria and restriction enzyme mediated cleavage of non viral sequence leaves a viral DNA substrate. Calcium phosphate based transfection into rhesus DF generates a pure population of recombinant virus. Amplification of recombinants in rhesus DF is carried out in tissue culture for evaluation *in vitro* and *in vivo*.

7.8 Assays

7.8.1. Assay for CCR7 Targeting of Virions/Cells to Secondary Lymph Organs

In vivo assays to measure increased targeting of virus (CMV) engineered to express CCR7 to secondary lymph organs can be performed as follows. Typically, the assays are carried out in an animal model, e.g., non-human primates, e.g., baboons or Rhesus monkeys (macaca mulatta).

In an exemplary assay, Rhesus CMV strain 68.1 (e.g., $10^4 - 10^7$ pfu, usually 10^6 pfu, in excipient) is administered by oral inoculation (or subcutaneous injection) to CMV-negative animals. "Experimental" animals, are inoculated with a recombinant CMV strain bearing CCR7, while "control" animals receive the parental "wild type" CMV strain Rh68.1.

The dissemination of $CCR7^+$ and $CCR7^-$ virus or virus infected cells to SLO is determined. Suitable assays for detecting this spread include gross measurements of SLO size or quantitative PCR of viral DNA. In one suitable assay, DNA isolated from SLO (e.g., using commercially available kits from Qiagen, CA) is assayed for viral DNA by PCR using CMV specific primers. For example, in one embodiment, DNA is purified from rhesus macaque SLO, then used as template for nested PCR with primers able to amplify the RhCMV immediate early 2 gene (5' GCC AAT GCA TCC TCT GGA TGT ATT GTG A 3' and 5' TGC TTG GGG AAT CTC TGC AC 3' then 5' CCC TTC CTG ACT ACT AAT GTA C 3' and 5' TTG GGG AAT CTC TGC ACA AG 3') (see, e.g., Tarantal et al., 1998, *J Infect Dis* 177:446-50). An increased titer of viral DNA in SLO tissues of animals infected with CCR7 knock in virus versus controls indicates CCR7 directed migration of virus or virus infected cells to SLO.

Migration can also be assayed by histology. Typically, tissue is fixed in paraformaldehyde and embedded in paraffin, or frozen in OCT for frozen sections (see, e.g., by Luna, L.G., THE MANUAL OF HISTOLOGIC STAINING METHODS OF THE ARMED FORCES INSTITUTE OF PATHOLOGY, , McGraw-Hill, 3rd edition, 1968). Sections are stained using an antibody specific for CMV (e.g., rhCMV). An increased number of cells expressing viral antigen in SLO tissues of animals infected with CCR7 knock in virus versus controls indicates CCR7 directed migration of virus or virus infected cells to SLO.

7.8.2. <u>Assay for induction of antigen (e.g., BAPA) specific IgG response</u>

In vivo assays in an animal model are sometimes used to demonstrate host response to expression of foreign antigens by recombinant CMVs. The assays can be carried out in humans, but are more typically conducted in non-human primates, e.g., Rhesus monkeys (macaca mulatta) or baboons, or other mammal models (e.g., mice).

In an exemplary assay, Rhesus CMV (e.g., $10^4 - 10^7$ pfu, usually 10^6 pfu, in excipient) is administered by oral inoculation (or subcutaneous injection) to CMV-negative animals. "Experimental" animals, are inoculated with a recombinant CMV strain bearing a gene encoding a foreign antigen (e.g., Bacillus anthracis protective antigen (BAPA)), while "control" animals receive the parental "wild type" CMV strain. At days 15 to 30 (preferably day 30) peripheral blood is drawn from inoculated animals and the serum tested for antibodies to antigen in a specific ELISA (e.g., for BAPA, the protocol of Little and Knudson, 1986, *Infection and Immunity* 52: 509-512 may be used).

7.8.3 <u>Assay for induction of antigen (e.g., BAPA) specific CD8 T cell response</u>

In vivo assays in an animal model are used to demonstrate host response to expression of foreign antigens by recombinant CMVs. The assays can be carried out in humans, but are more typically conducted in non-human primates, e.g., Rhesus monkeys (macaca mulatta) or baboons.

In an exemplary assay, Rhesus CMV (e.g., $10^4 - 10^7$ pfu, usually 10^6 pfu, in excipient) is administered by oral inoculation (or intravenous injection) to CMV-negative animals. "Experimental" animals, are inoculated with a recombinant CMV strain bearing a gene encoding a foreign antigen, while "control" animals receive the parental "wild type" CMV strain. At days 15 to 30 (preferably day 30) peripheral blood is drawn from inoculated animals.

Ficoll purified PBMC are obtained from experimental and control animals and are resuspended and placed in 96-well microtiter plates coated with anti-monkey IFN_ mAb MD-1 (U-Cytech, Utrecht, Netherlands). PBMC are plated in duplicate at two-fold dilutions ranging from 5 to 0.3×10^5 cells per well.

PBMC are infected with vaccinia recombinants expressing BAPA, as well as with control vaccinia at a moi of 10 PFU/cell at 37°C in a 5% CO_2 incubator. After 16 to 24 hours, cells are removed by extensive washing and the wells were serially incubated with a biotinylated anti-monkey IFN_ detector antibody (U-Cytech), followed by gold-labeled anti-biotin IgG (U-Cytech). An activator mix (U-Cytech) is added which allowed formation of silver salt precipitates at the sites of gold clusters. Spots are counted on a Zeiss ELISPOT imaging system. BAPA expressing vaccinia are constructed as previously described (Iacono-Connors L.C. et al , *Infection and Immunity* 58: 366-372, 1990).

7.8.4 Assays for Inhibition of CMV Dissemination by Gene Knock Out

Animal models can be used to demonstrate reduction of CMV dissemination when viral dissemination genes are disabled. Typically, *in vivo* assays in an animal model are used to demonstrate reduction of CMV. Typically, the assays are conducted in non-human primates, e.g., Rhesus monkeys (macaca mulatta) or baboons. In an exemplary assay, Rhesus CMV strain 68.1 (e.g., $10^4 - 10^7$ pfu, usually 10^6 pfu, in excipient) is administered by oral inoculation (or intravenous injection) to CMV-negative animals. "Experimental" animals, are inoculated with a recombinant CMV strain, while "control" animals receive the parental "wild type" CMV strain.

Rhesus is a favored model system for analysis. Rhesus CMV is able to replicate in human foreskin fibroblasts and conversely HCMV in primary chimpanzee fibroblasts (Perot et al., 1992, *J Gen Virol.* 73:3281-84) suggesting the close relatedness of the human and primate viruses. In addition certain of the inventors have shown that rhesus CMV is largely co-linear with HCMV, individual ORFs are largely homologous (50-65%) and peptides share up to 90% identity (Lockridge et al., 2000, *Virology* 268:272-80; Barry et al., 1996, *Virology* 215:61-72, 218:296). Furthermore the natural history of infection as well as characteristics of persistence and pathogenesis mirror those of HCMV (Lockridge et al., 1999, *J Virol.* 73:9576-83. Tarantal et al., 1998, *J Infect Dis.* 177:446-50).

Viral infection and dissemination in experimental and control animals is determined by analyzing spread of the virus from the site of primary inoculation. See Saederup et al, 1999, *Proc. Nat. Acad. Sci. USA* 96:10881-86. Suitable assays for detecting

CMV are described in Lockridge et al., 1999, *J Virol.* 73:9576-83. Sterile blood, saliva and urine samples are collected at the time of virus administration and thereafter periodically (e.g., every day or every 3 days) and assayed for virus. According to one suitable assay, viral titer is measured in saliva, urine and blood samples, by co-cultivation of serial dilutions of sterile samples with a cell permissive for CMV replication (e.g., rhesus dermal fibroblasts) for a period of about 2 weeks, and counting of viral plaques, using standard techniques (Spaete and Mocarski, 1985, *J Virol* 56:135-43). Inhibition of CMV dissemination is demonstrated by a 5-fold or greater (e.g., at least 10-fold) reduction in the overall titer of infectious virus in at least one, sometimes two or three of these fluids in experimental animals compared to control animals when assayed at timepoints after innoculation (e.g., 3, 6, 9, 12, or 15 days or 1 month following innoculation). Inhibition of CMV dissemination can alternatively be shown by a delay in appearance of detectable virus in at least one, sometimes two or three of the these fluids in experimental animals compared to control animals.

In another suitable assay, blood is assayed for viral DNA by PCR using CMV specific primers. For example, in one embodiment, DNA is purified from plasma (e.g., using commercially available kits from Qiagen, CA), then used as template for nested PCR with primers able to amplify the RhCMV immediate early 2 gene (5' GCC AAT GCA TCC TCT GGA TGT ATT GTG A 3' and 5' TGC TTG GGG AAT CTC TGC AC 3' then 5' CCC TTC CTG ACT ACT AAT GTA C 3' and 5' TTG GGG AAT CTC TGC ACA AG 3') (see, e.g., Tarantal et al., 1998, *J Infect Dis* 177:446-50). Inhibition of CMV dissemination is demonstrated by a difference of viral titer or kinetics (as described *supra*) as assessed by levels of viral DNA in peripheral blood.

Assays for viral dissemination can also be carried out by direct detection of CMV in tissues including lung, spleen thymus, salivary gland, bone marrow, pancreas, kidney, tonsil, liver, parotid gland, esophagus and others. Thus, in embodiment, animals are necropsied 15 to 30 days after administration of virus (e.g., day 30) and complete tissue and blood samples taken. DNA is purified from tissue (e.g. using commercially available kits from Qiagen, CA), then used as template for nested PCR as described *supra*. Dissemination can also be assayed by histology. Typically, tissue is fixed in paraformaldehyde and embedded in paraffin, or frozen in OCT for frozen sections (see, e.g., by Luna, L.G., THE MANUAL OF HISTOLOGIC STAINING METHODS OF THE ARMED FORCES INSTITUTE OF PATHOLOGY, McGraw-Hill, 3rd edition, 1968). Sections are stained using an antibody specific for CMV (e.g., rhCMV). Inhibition of CMV dissemination is demonstrated by a

difference of viral titer or kinetics (as described *supra*) as assessed by levels of viral antigens in specific tissues or organs of experimental animals compared to control animals.

In another suitable assay, levels of reactive leukocytes are assayed, e.g., by FACS analysis of blood samples. Suitable assays are described in Lockridge et al., *J Virol.* 73:9576-83, *supra*. Briefly, activated T cells are identified by dual fluorescent staining for CD3 (T cell marker, Pharmingen, clone SP34) and CD69 (very early activation marker, Becton Dickinson, clone L78) while memory T cells will be identified by dual fluorescent staining for CD3 (T cell marker, Pharmingen, clone SP34) and CD45RO (memory cell marker, Dako, clone UCHL1). Inhibition of CMV dissemination is demonstrated by fewer activated T cells or memory T cells in peripheral blood of experimental animals compared to control animals (e.g., at least about 30% or about 50% fewer, often at least 80% fewer when measured following administration (e.g., 3, 6, 9, 12, or 15 days or 1 month following administration of the agent).

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It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the claims. Each and every publication, patent, and patent application cited herein are hereby incorporated by reference in their entirety for all purposes.

SEQUENCES

SEQ ID NO:1

Nucleotide sequence for RhUS28.1

ATGAATAACACATCTTGCAACTTCAACGTCACTCTCAACGCATCGGCACCAAGCC
GATACATAGCTATTGCTATGTACAGCATTGTTATCTGTATCGGGTTGGTTGGAAA
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SEQ ID NO:2

Nucleotide sequence for RhUS28.2

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SEQ ID NO:3

Nucleotide sequence for RhUS28.3

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SEQ ID NO:4

Nucleotide sequence for RhU28.4

ATGAATTCGAGCCAGCACAACATAAGCGTGTTTCTCTCCATTGGAGCAGGGCCCG
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SEQ ID NO:5

Nucleotide sequence for RhUS28.5

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SEQ ID NO:6

Nucleotide sequence for RhUL33

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SEQ ID NO:7

Nucleotide sequence for RhUL33 spliced

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SEQ ID NO:8

Nucleotide sequence for RhUL78

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SEQ ID NO:9

CGGCCAAGATGTCCCAAGAGGTTCTGACATGAACAATCACTTTTC
CGAGATAGATGAGTTTGTTAGTGGCATTTACCAGAGAACTATTGGAGTGACGCT
CAAGATGAAGCTTCACTGGCCGTATTTCGAACATATTGTTAGATATAGCTAGTA
AAGAATCTTCTAAAGCCATGACGTCTTTCTGACGAAGTTGAATAAATTCTATCT
CACCAGTACCCAAAGGCTGACACTCAGACAACTTTGCCAAGGCCGTTGCACCCA
CCATGGCATTCTGAATCACAGTAACATCCGTCCGAGAATCGTCACCAAAAACGG
TGGCCTCCAAAGTTCGCAGGTGAGGCCGAGCCTTTACTGGATCTCGGAAGGGAT
ACATGTGTGCTCGCCGAGTGACAGCATTAGCATTAACCTCAAACTCATCTAAAA

GCGATGATAAATCAGGAATATGATAGCGCAATTCTCGATAGTAGGCCAACCAG AGGACTAATTGGTTGAACAGACAGCTCCGTCTGTGCAAAAACTTTTCGCCGCAT TTTCTGAGAATTTTAGGATGCTGCTCTAAATCTACGTTCTCTTTAGTCGGCAGGG TCTTTAAAAAGTTAGTGATGGCAGTCACTTTACGAGGCGGCAGCCCGATAAACT TTAAACTCATGATTGTCAGCCACAGAAACCGGAAATTTCACGAGATACGGCTGT TTCAGCGTTCTGCTATCCGTCCAGGCGGGTTATGGAAACCATTCTTCACAACCG AACGGTGAGTGACATTTAAGACAGTTTAATAGCCAACACTCGTAACGTCTCGGA AGCTGATAAGTTTCGTTTTTCCACAGAGTGAAACTAATTCCATTTTGCACATCAA CACCACCTGCAATGTGACCGACTCACTGTACGCCGCCAAACTAGGCGAAGCCCT CGTGAACAGCGCGCTAGCTTTATTCGGTACCCCCCTCAACGCCATCGTCCTCGT CACACAGCTATTGGCCAACCGAGTTCATGGATACTCCACCCCGATTATCTACAT GACCAATCTTTACTCTGCCAATTTTCTCACCTTGATAGTACTTCCTTTTATCGTTT TAAGCAATCAACACCTTTTACCTGCCAGTGCAGTAACCTGTAAATTTCTCTCCCT GTTGTACTACTCTAGCTGCAGCGTAGGTTTTGCTACAGTGGCACTGATAGCGGC CGACCGATACCGAGTGATTCATCGCCGAACTCAAGCTCGCCAATCCTACCGTAA CACATATATGATAGTAGGCTTAACGTGGCTCATTGGCTTGATCTGCGCTACCCC CGGGGGGGTCTACACAACCATTGTAGCTCACCGCGATGGGGAAAGTGATGCTC AAAGACACAATACTTGCATTATGCACTTTGCGTATGATGAAGTTTACGTCCTCA TGGTCTGGAAACTTCTCATCGTTTTAGTCTGGGGCATAGTGCCAGTTGTCATGAT GAGCTGGTTTTACGCGTTTTTTACAATACTGTACAAAGAACAGCCAAAAAACA ACAACGTACGTTGAAATTCGTAAAGGTATTACTCCTGTCATTCATCATCATCCA AACTCCCTATGTGTCAATCATGATTTTTAACACGTATGCCACCGTAGGATGGCC GATGGAATGCGCCGATCTAACTAGACGCCGAGTCATCAACACGTTTTCCCGTCT CGTCCCCAATCTACATTGCATGGTCAACCCCATCCTCTACGCTCTCATGGGAAA TGACTTTGTGTCTAAAGTGGGCCAATGCTTTCGGGGGGAACTCACGAACCGTCG AACTTTTCTGCGTTCCAAGCAACAAGCCCGCAACTCGGACGATGTACCGACAAT TGTCAGTCAACAACCCGCCACACCCACCATCGTCAATAAGCCCGAAAAAAACC CGCACGTAAAACGCGGTGTATCTTTCAGCGTCAGCGCATCTTCCGAACTCGCAG CGGCCAAAAAAGCCAAAGACAAAGCCAAGCGGCTTTCCATGTCCCACCAAAAC CTACGTCTGACGTGAATTTTCCTAGAGGCTGCCTCCACGGGTTTACATACATATC TCGGTACTTGCTACACTTGATCACTTTACTGCGGACACCACGGCCAATCGCATC

WHAT IS CLAIMED IS:

1. A recombinant cytomegalovirus (CMV), comprising a cytomegalovirus (CMV) genome which comprises a first heterologous nucleotide sequence encoding a heterologous chemokine element, and (ii) a second heterologous nucleotide sequence encoding an immunogenic polypeptide.

2. The recombinant CMV of claim 1, wherein the CMV genome is encapsulated in infectious form as a virion.

3. The recombinant CMV of claim 2, wherein the CMV genome is attenuated to reduce virulency in a host.

4. The recombinant CMV of claim 3, wherein a viral dissemination gene is disabled.

5. The recombinant CMV of claim 4, wherein the viral dissemination gene is a gene encoding a viral chemokine element or a viral immune-modulatory gene.

6. The recombinant CMV of claim 5, wherein the gene encoding the viral chemokine element is selected from the group consisting of US28, US27, UL33, UL78, UL146, UL147, MCK-1 and MCK-2, or a homolog thereof.

7. The recombinant CMV of claim 5, wherein the viral immune-modulatory gene is selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18, UL40, m144, m152, m04, m06 and m138, or a homolog thereof.

8. The recombinant CMV of claim 3, wherein the CMV genome is attenuated to reduce virulency in a mammal.

9. The recombinant CMV of claim 3, wherein the host is selected from the group consisting of a non-human primate and commercial livestock.

10. The recombinant CMV of claim 8, wherein the mammal is a rhesus monkey.

11. The recombinant CMV of claim 8, wherein the mammal is a mouse.

12. The recombinant CMV of claim 3, wherein the heterologous chemokine element is endogenous to the host.

13. The recombinant CMV of claim 12, wherein the heterologous chemokine element is a chemokine ligand.

14. The recombinant CMV of claim 12, wherein the heterologous chemokine element is a chemokine receptor.

15. The recombinant CMV of claim 12, wherein the CMV genome is attenuated to reduce virulency in a mammal and the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

16. The recombinant CMV of claim 2, wherein the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen.

17. The recombinant CMV of claim 16, wherein the pathogenic organism is a bacterium, a virus or a parasite.

18. The recombinant CMV of claim 17, wherein the immunogenic polypeptide comprises a fragment of a polypeptide from organisms selected from the group consisting of Bacillus anthracis, Dengue, Yersinia pestis, Ebola, Marburg, Lassa, Venezulean Equine Encephalitis and Eastern Equine Encephalitis.

19. The recombinant CMV of claim 16, wherein the immunogenic polypeptide comprises a tumor antigen.

20. The recombinant CMV of claim 19, wherein the tumor antigen is selected from the group consisting of antigens associated with breast cancers, lung cancers, thryroid carcinomas, squamous cell carcinomas and renal cell carcinomas.

21. The recombinant CMV of claim 3, wherein the first and second heterologous nucleotide sequence are operably linked to a promoter that is operative in the host.

22. The recombinant CMV of claim 21, wherein the first and second heterologous nucleotide sequence are operably linked to different promoters that are operative in the host.

23. The recombinant CMV of claim 3, wherein the construct is formulated as a composition, the composition comprising the construct and a pharmaceutically acceptable adjuvant, carrier, diluent or excipient.

24. The recombinant CMV of claim 2, wherein

(a) the immunogenic polypeptide comprises an antigen from an organism that is pathogenic in humans or a human tumor antigen;

(b) a viral chemokine element or a viral immune-modulatory gene is disabled, the viral chemokine element selected from the group consisting of US28, US27, UL33, UL78, UL146 and UL 147 and the viral immune-modulatory gene selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18 and UL40; and

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

25. The recombinant CMV of claim 2, wherein

(a) the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen;

(b) a viral chemokine element or a viral immune-modulatory gene is disabled, the viral chemokine element selected from the group consisting of rhUS28.1, rhUS28.2, rhUS28.3, rhUS28.4, rhUS28.5, rhUL33 and rhUL78 and the viral immune-modulatory gene selected from the group consisting of rhUL111A, US3, US6, US11, US2, UL83 and UL40; and

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

26. The recombinant CMV of claim 2, wherein

(a) the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen;

(b) a viral chemokine element or a viral immune-modulatory gene is disabled, the viral chemokine element selected from the group consisting of MUL33, MUL78, MCK-1 and MCK-2 and the viral immune-modulatory gene selected from the group consisting of m144, m152, m04, m06, m138; and

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

27. A recombinant cytomegalovirus (CMV) comprising a cytomegalovirus (CMV) genome that comprises a heterologous nucleotide sequence encoding a heterologous chemokine receptor or ligand.

28. The recombinant CMV of claim 27, wherein the CMV genome is attenuated to reduce virulency in a host and encapsulated in infectious form.

29. The recombinant CMV of claim 28, wherein the CMV genome is attenuated by virtue of a disabled viral dissemination gene and/or a viral immune-modulatory gene.

30. The recombinant CMV of claim 27, wherein the chemokine element is endogenous to the host.

31. A recombinant CMV comprising a cytomegalovirus (CMV) genome that comprises a heterologous nucleotide sequence encoding an immunogenic polypeptide.

32. The recombinant CMV of claim 31, wherein the CMV genome is attenuated to reduce virulency in a host and encapsulated in infectious form.

33. The recombinant CMV of claim 32, wherein the CMV genome is attenuated by virtue of a disabled viral dissemination gene and/or a viral immune-modulatory gene.

34. The recombinant CMV of claim 31, wherein immunogenic polypeptide comprises an antigen from an organism that is pathogenic in a host or a tumor antigen.

35. A method for inducing an immune response in a host, the method comprising administering a composition to a host, wherein the composition comprises a recombinant cytomegalovirus (CMV) with a genome that contains (i) a first heterologous nucleotide sequence encoding a heterologous chemokine element, and (ii) a second heterologous nucleotide sequence encoding an immunogenic polypeptide.

36. The method of claim 35, wherein the CMV genome is attenuated to reduce virulency in the host.

37. The method of claim 36, wherein a viral dissemination gene is disabled.

38. The method of claim 37, wherein the viral dissemination gene is a gene encoding a viral chemokine element or a viral immune-modulatory gene.

39. The method of claim 38, wherein the gene encoding the viral chemokine element is selected from the group consisting of US28, US27, UL33, UL78, UL146, UL147, MCK-1 and MCK-2, or a homolog thereof.

40. The method of claim 38, wherein the viral immune-modulatory gene is selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18, UL40, m144, m152, m04, m06 and m138, or a homolog thereof.

41. The method of claim 35, wherein the heterologous chemokine element is endogenous to the host.

42. The method of claim 41, wherein the heterologous chemokine element is a chemokine ligand.

43. The method of claim 41, wherein the heterologous chemokine element is a chemokine receptor.

44. The method of claim 41, wherein the host is a mammal and the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or homolog thereof.

45. The method of claim 35, wherein the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen.

46. The method of claim 45, wherein the pathogenic organism is a bacterium, a virus or a parasite.

47. The method of claim 35, wherein

(a) the host is a human;

(b) a viral chemokine element or a viral immune-modulatory gene is disabled, the viral chemokine element selected from the group consisting of US28, US27, UL33, UL78, UL146 and UL147 and the viral immune-modulatory gene selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18 and UL40;

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof; and

(d) the immunogenic polypeptide comprises an antigen from an organism that is pathogenic in humans or a human tumor antigen.

48. The method of claim 35, wherein

(a) the host is a rhesus monkey;

(b) a viral chemokine element or a viral immune-modulatory gene is disabled, the viral chemokine element selected from the group consisting of rhUS28.1, rhUS28.2, rhUS28.3, rhUS28.4, rhUS28.5, rhUL33 and rhUL78 and the viral immune-modulatory gene selected from the group consisting of rhUL111A, US3, US6, US11, US2, UL83 and UL40;

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof; and

(d) the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen.

49. The method of claim 35, wherein

(a) the host is a mouse;

(b) a viral chemokine element or a viral immune-modulatory gene is disabled, the viral chemokine element selected from the group consisting of MUL33, MUL78, MCK-1 and MCK-2 and the viral immune-modulatory gene selected from the group consisting of m144, m152, m04, m06, m138;

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof; and

(d) the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen.

50. A therapeutic or prophylactic treatment method, the method comprising administering a composition to an animal, wherein

(a) the composition comprises an attenuated recombinant cytomegalovirus (CMV) with a genome that contains (i) a first nucleotide sequence encoding a chemokine receptor or chemokine that is endogenous to the animal, and (ii) a second nucleotide sequence encoding an immunogenic polypeptide; and

(b) the immunogenic polypeptide comprises an antigen correlated with a disease or infection which the animal has or is susceptible to obtaining; and whereby

the administered composition induces an immune response in the animal.

51. The method of claim 50, wherein the CMV genome is attenuated by virtue of a disabled viral dissemination gene.

52. The method of claim 51, wherein the viral dissemination gene is a gene encoding a viral chemokine element or a viral immune-modulatory gene.

53. The method of claim 52, wherein the gene encoding the viral chemokine element is selected from the group consisting of US28, US27, UL33, UL78, UL146, UL147, MCK-1 and MCK-2, or a homolog thereof.

54. The method of claim 52, wherein the viral immune-modulatory gene is selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18, UL40, m144, m152, m04, m06 and m138, or a homolog thereof.

55. The method of claim 50, wherein the animal is a mammal.

56. The method of claim 50, wherein the animal is selected from the group consisting of a human, a non-human primate and commercial livestock.

57. The method of claim 55, wherein the mammal is selected from the group consisting of a non-human primate and a mouse.

58. The method of claim 50, wherein

(a) the animal is a human;

(b) the CMV genome is attenuated by virtue of a disabled viral chemokine element or a viral immune-modulatory gene, the viral chemokine element selected from the group consisting of US28, US27, UL33, UL78, UL146 and UL147 and the viral immune-modulatory gene selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18 and UL40; and

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

59. The method of claim 50, wherein

(a) the animal is a rhesus monkey;

(b) the CMV genome is attenuated by virtue of a disabled viral chemokine element or a viral immune-modulatory gene, the viral chemokine element selected from the group consisting of rhUS28.1, rhUS28.2, rhUS28.3, rhUS28.4, rhUS28.5, rhUL33 and rhUL78 and the viral immune-modulatory gene selected from the group consisting of rhUL111A, US3, US6, US11, US2, UL83 and UL40; and

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

60. The method of claim 50, wherein

(a) the animal is a mouse;

(b) the CMV genome is attenuated by virtue of a disabled viral chemokine element or a viral immune-modulatory gene, the viral chemokine element selected from the group consisting of MUL33, MUL78, MCK-1 and MCK-2 and the viral immune-modulatory gene selected from the group consisting of m144, m152, m04, m06, m138; and

(c) the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or a homolog thereof.

61. A method of preparing a recombinant CMV, comprising inserting into a CMV genome (i) a first heterologous nucleotide sequence encoding a heterologous chemokine element, and (*ii*) a second heterologous nucleotide sequence encoding an immunogenic polypeptide.

62. The method of claim 61, further comprising attenuating the CMV genome to reduce virulency in a host.

63. The method of claim 62, wherein attenuation comprises disabling a viral dissemination gene.

64. The method of claim 63, wherein the viral dissemination gene is a gene encoding a viral chemokine element or a viral immune-modulatory gene.

65. The method of claim 64, wherein the gene encoding the viral chemokine element is selected from the group consisting of US28, US27, UL33, UL78, UL146, UL147, MCK-1 and MCK-2, or a homolog thereof.

66. The method of claim 64, wherein the viral immune-modulatory gene is selected from the group consisting of UL111A, US3, US6, US11, US2, UL83, UL18, UL40, m144, m152, m04, m06 and m138, or a homolog thereof.

67. The method of claim 61, wherein the CMV genome is attenuated to reduce virulency in a mammal.

68. The method of claim 62, wherein the host is selected from the group consisting of a human, a non-human primate and commercial livestock.

69. The method of claim 67, wherein the mammal is selected from the group consisting of a rhesus monkey and a mouse.

70. The method of claim 62, wherein the heterologous chemokine element is endogenous to the host.

71. The method of claim 70, wherein the heterologous chemokine element is a chemokine ligand.

72. The method of claim 70, wherein the heterologous chemokine element is a chemokine receptor.

73. The method of claim 70, wherein the heterologous chemokine element is selected from the group consisting of MIP3α, SLC, MDC, MC10, MIP1β, ELC and CCR7, or homolog thereof.

74. The method of claim 62, wherein the immunogenic polypeptide comprises an antigen from a pathogenic organism or a tumor antigen.

75. The method of claim 74, wherein the pathogenic organism is a bacterium, a virus or a parasite.

76. The method of claim 61, further comprising inserting at least one promoter that is operative in the host into the CMV genome such that the at least one promoter is operably linked to the first and second heterologous nucleotide sequence.

77. The method of claim 61, further comprising inserting at least two promoters that are operative in the host into the CMV genome such that the first and second heterologous nucleotide sequence are operably linked to different promoters.

78. The method of claim 61, further comprising combining the recombinant CMV of claim 1 with a pharmaceutically acceptable carrier, diluent or excipient.

RhUS28.1

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        10         20         30         40         50         60
ATGAATAACA CATCTTGCAA CTTCAACGTC ACTCTCAACG CATCGGCACC AAGCCGATAC

        70         80         90        100        110        120
ATAGCTATTG CTATGTACAG CATTGTTATC TGTATCGGGT TGGTTGGAAA CCTGCTGTTA

       130        140        150        160        170        180
TGCATCGTGT TAGTCAAGAA ACGCAAACTG CGATATTCCA GCGATGTTTA TTTTTTCCAC

       190        200        210        220        230        240
GCCTCTATGG CCGACCTCGT CAGCACTGTC ATGCTACCGC TCTGGCTACA TTATGTCCTC

       250        260        270        280        290        300
AACTTTGCCC AACTCTCTCG AGGAGCCTGT ATCAGCTTTT CGGTGACTTT CTATGTTCCC

       310        320        330        340        350        360
CTTTTCGTTC AGGCCTGGTT ACTCATTTCC ATCGCTATGG AGCGATATTC CAACTTAGTA

       370        380        390        400        410        420
TGGATGGCAC CCATTAGCGT TAAGACGGCC TTTAAACACT GCATAGGAAC CTGGATCGTA

       430        440        450        460        470        480
TCTGCCTTCG TGGCATCACC CTACTACGCA TACAGAAACT CACACGACGA ACACGAATGC

       490        500        510        520        530        540
ATTCTAGGAA ACTACACTTG GCACATTAAC GAACCGCTAC ACACGTGTAT GGATGTGGTG

       550        560        570        580        590        600
ATCATAGTAT GGACCTTTTT GGCCCCAGTA CTGGTAACCA TTATAGCAAG CGTCAAAATG

       610        620        630        640        650        660
AGACGAACGA CCTGGGGCAA TACTAGGTTA AACGAAAAGA ACAGCGACAT TCTTATAGTA

       670        680        690        700        710        720
CTAGTTGTCA TGACAGTGTT CTTTTGGGGA CCGTTTAATA TCGTGTTGGT TATTGACAAT

       730        740        750        760        770        780
ATTTTACAGA GATACTATGA TACCACGAAT TGCGATGTAG AAAAGATTAA ACATATCATG

       790        800        810        820        830        840
GCTATGATCT CAGAAGCCAT TGTTTATTTT CGCGGTATTA CAGCACCTAT TATTTATGTA

       850        860        870        880        890        900
GGGATTAGTG GCAGATTTCG CGAAGAGATT TACTCTCTGT TTAGACGCCA GCCGTATAAC

       910        920        930        940        950        960
GATTTGGACC CCGATGCCAA TCAATTCATG ATTGAACTCA CTAGCCAGGG AAGAAGTAGA

       970        980        990       1000       1010       1020
AATAGAAATG CTAGACAATC GGAAAGCAAT GTACCGCAAC CAGAAGAATG CTTCTGGTAA

      1030       1040       1050       1060       1070       1080
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FIG. 1

RhUS28.2

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        10         20         30         40         50         60
ATGACCAACG CCGGACACTG TCACATAAAC GAAAGTCTCG CGTCGTATGG AATCGCTCCC

        70         80         90        100        110        120
GCAGCTACCA TTACCTTATA CAGCATTGCG GGAATCTGCG GTGTCACGGG AAATCTGTTA

       130        140        150        160        170        180
ATACTTTTGG TTTTGTTCAC GAGACGCATA CACTGGTTCG CAAATGACAT CTACTATCTC

       190        200        210        220        230        240
AACATGATCT TTACAGACTT TCTTGTTTTC ATTACATTAC CCGCCTGGGT TTACTACCTG

       250        260        270        280        290        300
CTGAATTACA CACAACTCTC ACACTATGCC TGCATTGCTC TATCATTTGT TTTTTACGTT

       310        320        330        340        350        360
TCCATTTTTA TTCAAGCTGA CTTTATGGTA GCAGTGGCTA TCGAGCGTTA TCGAAGCCTA

       370        380        390        400        410        420
GTGAAAAACA AACCCCTTAG CGTAAAAAAA GCCAGCGTCA GCTGCGCGTG CATCTGGATC

       430        440        450        460        470        480
ATTGTTATTA TAGTGTCTTC ACCATACTAC ATGTTTAGAT CGCAACACGA AACAAATTCT

       490        500        510        520        530        540
TGCATTCTAG GAAACTACAC CTGGCATATG AACAGTCCTT TTCGCACCAC AATGGACGCA

       550        560        570        580        590        600
TCCATTAACA TTTGGTCTTT TGTCGTTCCG GCCGTGACGA CCTTGTTAAT AGCCAGACGA

       610        620        630        640        650        660
ATTTATGTAT GTACTTCAGG CAACAAAAAA ATGAACGCCA GAGCCAGTGG TTTGTTAGAG

       670        680        690        700        710        720
GCCATGGTGA TTAGCATGTT ATTCTTCGGA GGACTTTTCA ACCTGAACAT CTTTCGAGAC

       730        740        750        760        770        780
ATAGTTTCGG ACACATCGGA AGACAATAAA GACTGCACAT ATCTTAAGCA GGAACACTTT

       790        800        810        820        830        840
ATTCGCATGG TCGGTGTGGC CCTCGTTTAC GGGCGCGCTA TATTCAACCC TTTTATGTAT

       850        860        870        880        890        900
ATGTGTGTGA GTACCAGATT GCGCCAAGAA ATAAAATGTT TGTTTATGCG AATACCTTAT

       910        920        930        940        950        960
GAAACACTAG ATGCAGAACA CGCTAAACTC ATGGTTAATT TAAAAAACAG AAATGCTAAT

       970        980        990       1000       1010       1020
GTACCCGATC CTAAACCTCG TGAATATGAA TCTGTGTTAT AG........ ..........
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FIG. 2

RhUS28.3

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        10         20         30         40         50         60
ATGACCAACA CTAACAATAC GACTTGTCAT CTCAACGGAA CTTTCGAAAC TTTTAAAATC

        70         80         90        100        110        120
ACCCGTCCAG TAGCCATCAG CGCCTACACT GTACTCGTGG TTATCGGACT TTTGGGAAAC

       130        140        150        160        170        180
ATTGTGCTGC TCAGCGTGCT CGTCGTGAAA CGCAAGCTCA AGTTTCCGAA TGACATTTAC

       190        200        210        220        230        240
TTTTTCAACG CGTCTTTGGC AGACGTTTTT GCCGTCTGCA TGTTGCCCGC CTGGGTTAAC

       250        260        270        280        290        300
TATGCACTGG ACTCCACACA ACTTAGCAAG TTCTCATGTA TCACTTTTAC GTTTGGTTTT

       310        320        330        340        350        360
TACGTCTCCC TGTTCATCCA GGCCTGGATG CTCATTCTGG TCACCCTGGA GCGATACGGA

       370        380        390        400        410        420
TCTCTAGTCT GGATCGCCCC GATCACCAGA AACAAAGCCA TAGCGAATTG TGTACTCTTT

       430        440        450        460        470        480
TGGCTTGTTT CCATCTTCTT GGCCGCACCT TACTACTCTT TTAGAAACGA AAGCAACGAA

       490        500        510        520        530        540
CACCAATGCA TCATGAGAAA CTATACCTGG AGCGTTGGTG AAACATGGCA CATAGCCCTG

       550        560        570        580        590        600
GATTTCTTAA TTACGCTCAT TACATTTATC ATGCCAGTGA CTATTGTGTT AGCTCTGAGT

       610        620        630        640        650        660
TTCAAAATGG CCAGATGGTC AACCTTTGGT TACAGAAACC TCACCAGCAG AACCAGTCTT

       670        680        690        700        710        720
ATCCTTATTT TGATACTGAC AGTAGCAGCA GGGTTCTGGG GACCTTTTCA CCTATTTATG

       730        740        750        760        770        780
TTTATAGAAA ACGTGGCAGG GCAGATTTAC CACATTCAAA AGGATTGCTG GTACTTACAG

       790        800        810        820        830        840
CTCAGACACT TGTGTAGCTT GATGACCGAA ACCCTAGTGT TTCTACGTTC AGTTTTTAAC

       850        860        870        880        890        900
CCTTATATTT ATATGATAAT CAGTTACAAG TTTAGGCAGC AGGTGCGCAG TCTACTCAAG

       910        920        930        940        950        960
CGTACTCAGT ATGATGCTTT GGACACGACT CAGTTAGCAG AAACTATGCA GCTGAAAGCG

       970        980        990       1000       1010       1020
AAAGGTGTGC CGGTGTCCGA CCCCGCGCCG CATGACTGCG AATGCTTTTT GTAA......
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FIG. 3

RhUS28.4

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        10         20         30         40         50         60
GAATTCGAGC CAGCACAACA TAAGCGTGTT TCTCTCCATT GGAGCAGGGC CCGTCATTAC

        70         80         90        100        110        120
CGGATACACG TGCGTTTTTC TGTTCGGGAT TCTGGGACAC TTTTACTTGT ATTGGAAAAA

       130        140        150        160        170        180
CCATCAGAGA CGACACCGGA CAAACAGTTT CAGTGATGTT TTATTTCGAC ATCTCATGAT

       190        200        210        220        230        240
CACCGAAGAG GTCTTTACCC TCACCATTCC CGTCTGGGCG TATCACTTAA CTACTCACGG

       250        260        270        280        290        300
CAACTTACCG GGCTCGTGGT GCCGAAGTCT CACCTTCGTT TTTTATCTAA CGGTATTCGC

       310        320        330        340        350        360
TCGTGCCTTC TTTTACCTGC TCCTCATCTG GGACCGATAC AGCGTAATCA TCTGCAGACA

       370        380        390        400        410        420
CCCTCTCCCC GTTAATCTGA ACTACAGTCA GGTCATAGGC CTGTCTGTCT GGCTGGTTGC

       430        440        450        460        470        480
CGTACTGTCA GCATCACCGT TCTCCATTTT TAACGGAAGT GTGAAACAAT GCCTGGGCAA

       490        500        510        520        530        540
CATGGGCAGC ATACCCAGCG AATCGTCTGC CGTTCTTAAC CTGGAAGTGC ACCTGTGCTC

       550        560        570        580        590        600
CTTCTGGTTA CCGCTCATCA TGTCGGCTAA CTGTTACTAC CAAGCAAAAC GCCGAGCATC

       610        620        630        640        650        660
GCCTGACCAA CTCCACGAAC TTTACCGATG CAGTTTGCTA ATTACCATTA TCACAACTTA

       670        680        690        700        710        720
CGCTATCGTA TGGTTTCCTT TCCATCTCGC TTTACTCATA GACGCCCTGA TTAGCATAAG

       730        740        750        760        770        780
CCATGTAGAA CCCTCTAGCG CTCTCCACTG GGCATCCATT GTCGTTACCT GTAAATCATT

       790        800        810        820        830        840
TACATTTGTA TATGCGGGCA TAAGCCCACT AGTGTATTTC ACATGCTGCC CCACCGTACG

       850        860        870        880        890        900
TCGCGAACTG CTGATGTCTC TACGTCCATT CTTCACCTGG ATTTCCAGCA AAACGCGGCG

       910        920        930        940        950        960
AGGCTACGCT CCGATTAAAA CACAACCTTT AAACATCCCC GACGAGCCGA TAGATAACAA

       970        980        990       1000       1010       1020
GTCACCGCAC CTGTTAAACG AATAA..... .......... .......... ..........
```

FIG. 4

RhUS28.5

10	20	30	40	50	60
ATGACTACCA	CCACAATGAG	TGCTACCACG	AATTCCAGTA	CCACGCCTCA	AGCAAGCAGC
70	80	90	100	110	120
ACCACGATGA	CAACGAAGAC	AAGCACTCCT	GGCAATACAA	CTACTGGCAC	TACGTCCACC
130	140	150	160	170	180
CTGACAACGA	TATCAACAAC	TTCTAATGCT	ACCAGCATAA	CGTCTAATTT	AAGCACTACC
190	200	210	220	230	240
GGAAACCAAA	CTGCAACTAC	CAATGCTACT	ACCTTCAGTT	CCACATTAAC	AACATCTACA
250	260	270	280	290	300
AATATAAGCA	GTACATTTTC	GACAGTTTCT	ACCGTCGCAT	CCAATGCAAC	ATGTAATTCT
310	320	330	340	350	360
ACAATCACAA	CGAATATTAC	AACTGCTTTT	ACTACAGCAG	CAAACACTAC	CGCAAGCAGC
370	380	390	400	410	420
CTCACCAGCA	TCGTAACTTC	ACTTGCCACT	ACCATTGAAA	CCACATCATT	TGATTATGAT
430	440	450	460	470	480
GAGTCAGCAG	AAGCTTGCAA	CTTAACAGAC	ATCGTTCATA	CTACTAGATC	AGTGACAGTT
490	500	510	520	530	540
ACTTTCTATA	CTATCATATT	CATACTCGGC	CTTTTGGGAA	ACTTTCTGGT	TCTTATGACC
550	560	570	580	590	600
ATCATTTGGA	ACCGTCGCAT	TTCCTTTATG	GTTGAAATAT	ATTTCGTTAA	TCTAGCAATC
610	620	630	640	650	660
TCCGATCTTA	TGTTTGTATG	TACTTTACCA	TTTTGGATAA	TGTATCTTCT	TGAGCACGAC
670	680	690	700	710	720
GTCATGTCAC	ATGCATCCTG	TGTAGCAATG	ACAGCCATTT	TTTATTGCGC	GCTGTTTGCC
730	740	750	760	770	780
AGCACTGTTT	TCCTCTTGCT	AATTGTTTTA	GACAGATGTT	ACGCTATTCT	ATTAGGTACA
790	800	810	820	830	840
GAAAAAGCAA	ATAGACGTTT	ATTGCGCAAT	GCTGTTTCTG	GATGCATGCT	CATGTGGGGA
850	860	870	880	890	900
TTGTGTTTCA	TTTTAGCATT	ACCTCATTTT	ATCTTTATGA	AGAAAGGAAC	CAACGTATGT
910	920	930	940	950	960
GTAGCAGAGT	ATGAACCAGG	ACTTAACAAT	TTCTATGTTA	TTTTTATCAA	TACTGAGGTG
970	980	990	1000	1010	1020
AACCTATGCA	CCCTAGTTTT	GCCAGCCGCA	GCCATTATCT	ACTGGTATCT	TAAACTAACC
1030	1040	1050	1060	1070	1080
AAAGCACTCA	AAACCCATGA	ACGACTGCGT	CATAGGCTAA	CGTCTCTAAA	CATAGTGTTA
1090	1100	1110	1120	1130	1140
GCTGTTGTCA	TTGTATTTGC	TTTGTTTTGG	CTGCCGTATA	ATCTCATGCT	TATGATGTAT
1150	1160	1170	1180	1190	1200
AGCTTAGTTC	ACATGCAGAT	ACCTTGGGAA	TGCAGCTCTG	AAAAAATACT	GAGACGAAGT
1210	1220	1230	1240	1250	1260
TTAATTATTA	CAGAATCCAT	CGCCCTCAGT	CACTGTTGCA	TCAACCCCAT	TATCTACTTG

FIG. 5A

RhUS28.5

1270	1280	1290	1300	1310	1320
CTCTTCGGAC	CTCGCTGTCG	AAGCGAGTTC	TGTCACCTGT	TGCGATGTTG	CTTTACGCGC
1330	1340	1350	1360	1370	1380
TTATGTCCAC	ACAGATCCTG	GAGTTCCATA	CGTGCAGAGA	CGGTGTCCAT	CAGTCTCAGT
1390	1400	1410	1420	1430	1440
CACTCACAGG	TATCTGCATC	ATCTGAGGAT	GATGACAACG	ATGTGCATGA	TGAATTGCAA
1450	1460	1470	1480	1490	1500
TTTTTAATTT	GA........	....	....	....	....

FIG. 5B

RhUL33

```
        10         20         30         40         50         60
ATGACCAATC TTTACTCTGC CAATTTTCTC ACCTTGATAG TACTTCCTTT TATCGTTTTA

        70         80         90        100        110        120
AGCAATCAAC ACCTTTTACC TGCCAGTGCA GTAACCTGTA AATTTCTCTC CCTGTTGTAC

       130        140        150        160        170        180
TACTCTAGCT GCAGCGTAGG TTTTGCTACA GTGGCACTGA TAGCGGCCGA CCGATACCGA

       190        200        210        220        230        240
GTGATTCATC GCCGAACTCA AGCTCGCCAA TCCTACCGTA ACACATATAT GATAGTAGGC

       250        260        270        280        290        300
TTAACGTGGC TCATTGGCTT GATCTGCGCT ACCCCCGGGG GGGTCTACAC AACCATTGTA

       310        320        330        340        350        360
GCTCACCGCG ATGGGGAAAG TGATGCTCAA AGACACAATA CTTGCATTAT GCACTTTGCG

       370        380        390        400        410        420
TATGATGAAG TTTACGTCCT CATGGTCTGG AAACTTCTCA TCGTTTTAGT CTGGGGCATA

       430        440        450        460        470        480
GTGCCAGTTG TCATGATGAG CTGGTTTTAC GCGTTTTTTT ACAATACTGT ACAAAGAACA

       490        500        510        520        530        540
GCCAAAAAAC AACAACGTAC GTTGAAATTC GTAAAGGTAT TACTCCTGTC ATTCATCATC

       550        560        570        580        590        600
ATCCAAACTC CCTATGTGTC AATCATGATT TTTAACACGT ATGCCACCGT AGGATGGCCG

       610        620        630        640        650        660
ATGGAATGCG CCGATCTAAC TAGACGCCGA GTCATCAACA CGTTTTCCCG TCTCGTCCCC

       670        680        690        700        710        720
AATCTACATT GCATGGTCAA CCCCATCCTC TACGCTCTCA TGGGAAATGA CTTTGTGTCT

       730        740        750        760        770        780
AAAGTGGGCC AATGCTTTCG GGGGGAACTC ACGAACCGTC GAACTTTTCT GCGTTCCAAG

       790        800        810        820        830        840
CAACAAGCCC GCAACTCGGA CGATGTACCG ACAATTGTCA GTCAACAACC CGCCACACCC

       850        860        870        880        890        900
ACCATCGTCA ATAAGCCCGA AAAAAACCCG CACGTAAAAC GCGGTGTATC TTTCAGCGTC

       910        920        930        940        950        960
AGCGCATCTT CCGAACTCGC AGCGGCCAAA AAAGCCAAAG ACAAAGCCAA GCGGCTTTCC

       970        980        990       1000       1010       1020
ATGTCCCACC AAAACCTACG TCTGACGTGA .......... .......... ..........
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FIG. 6

RhUL33 spliced

```
        10         20         30         40         50         60
ATGGCAGTCA CTTTACGAGG CGGCAGCCCG ATAAACTTTA AACTCATGAT TGTCAGCCAC

        70         80         90        100        110        120
AGAAACCGGA AATTTCACGA GATACGGCTG TTTCAGCGTT CTGCTATCCG TCCAGGCGGG

       130        140        150        160        170        180
TTATGGAAAC CATTCTTCAC AACCGAACG- ---------- ---------- ----------

       190        200        210        220        230        240
---------- ---------- ---------- ---------- -----AGTGA AACTAATTCC

       250        260        270        280        290        300
ATTTTGCACA TCAACACCAC CTGCAATGTG ACCGACTCAC TGTACGCCGC CAAACTAGGC

       310        320        330        340        350        360
GAAGCCCTCG TGAACAGCGC GCTAGCTTTA TTCGGTACCC CCCTCAACGC CATCGTCCTC

       370        380        390        400        410        420
GTCACACAGC TATTGGCCAA CCGAGTTCAT GGATACTCCA CCCCGATTAT CTACATGACC

       430        440        450        460        470        480
AATCTTTACT CTGCCAATTT TCTCACCTTG ATAGTACTTC CTTTTATCGT TTTAAGCAAT

       490        500        510        520        530        540
CAACACCTTT TACCTGCCAG TGCAGTAACC TGTAAATTTC TCTCCCTGTT GTACTACTCT

       550        560        570        580        590        600
AGCTGCAGCG TAGGTTTTGC TACAGTGGCA CTGATAGCGG CCGACCGATA CCGAGTGATT

       610        620        630        640        650        660
CATCGCCGAA CTCAAGCTCG CCAATCCTAC CGTAACACAT ATATGATAGT AGGCTTAACG

       670        680        690        700        710        720
TGGCTCATTG GCTTGATCTG CGCTACCCCC GGGGGGGTCT ACACAACCAT TGTAGCTCAC

       730        740        750        760        770        780
CGCGATGGGG AAAGTGATGC TCAAAGACAC AATACTTGCA TTATGCACTT TGCGTATGAT

       790        800        810        820        830        840
GAAGTTTACG TCCTCATGGT CTGGAAACTT CTCATCGTTT TAGTCTGGGG CATAGTGCCA

       850        860        870        880        890        900
GTTGTCATGA TGAGCTGGTT TTACGCGTTT TTTTACAATA CTGTACAAAG AACAGCCAAA

       910        920        930        940        950        960
AAACAACAAC GTACGTTGAA ATTCGTAAAG GTATTACTCC TGTCATTCAT CATCATCCAA

       970        980        990       1000       1010       1020
ACTCCCTATG TGTCAATCAT GATTTTTAAC ACGTATGCCA CCGTAGGATG GCCGATGGAA

      1030       1040       1050       1060       1070       1080
TGCGCCGATC TAACTAGACG CCGAGTCATC AACACGTTTT CCCGTCTCGT CCCCAATCTA

      1090       1100       1110       1120       1130       1140
CATTGCATGG TCAACCCCAT CCTCTACGCT CTCATGGGAA ATGACTTTGT GTCTAAAGTG

      1150       1160       1170       1180       1190       1200
GGCCAATGCT TTCGGGGGGA ACTCACGAAC CGTCGAACTT TTCTGCGTTC CAAGCAACAA

      1210       1220       1230       1240       1250       1260
GCCCGCAACT CGGACGATGT ACCGACAATT GTCAGTCAAC AACCCGCCAC ACCCACCATC
```

FIG. 7A

RhUL33 spliced

```
      1270       1280       1290       1300       1310       1320
GTCAATAAGC CCGAAAAAAA CCCGCACGTA AAACGCGGTG TATCTTTCAG CGTCAGCGCA

      1330       1340       1350       1360       1370       1380
TCTTCCGAAC TCGCAGCGGC CAAAAAAGCC AAAGACAAAG CCAAGCGGCT TTCCATGTCC

      1390       1400       1410       1420       1430       1440
CACCAAAACC TACGTCTGAC GTGA...... .......... .......... ..........
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FIG. 7B

RhUL78

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        10         20         30         40         50         60
ATGATTACGG AGCGCGTCCT CGCAGGCATC CTCGCGGGCA TGACGGCCGC GGGGAGTTTG

        70         80         90        100        110        120
GTCATTCTCC TCGCGGTTGT TATGTGGTTG AACATGTTAG ATCGCGCTGG CATGCCAATG

       130        140        150        160        170        180
GCCGTTGGGC ATTACACAGG GAACCTGGTG TTGACTCAGG TCATCTGTAT CTTCTCCATG

       190        200        210        220        230        240
CTGGCGTCTA AAATTGTTGG CATGACGAGT GCGGCCAACA TGGGCTTCTG CGGCATCGTG

       250        260        270        280        290        300
GTTTTTCTGG AAGACACTGG CCTCTATGTC ACCTCGCTGC TCTTCATGTT TATGATCCTG

       310        320        330        340        350        360
GATCGCATGG CGGCTTTTCT TAACGGGCGT CTTTTCTGGA GGCAGCAGAC GACGAAGCAG

       370        380        390        400        410        420
AATCTGAGTA CAAGCGTGTA CATTATTCTG TTTTGCTGGG TGTTGGGAAT GGCCGCGGCT

       430        440        450        460        470        480
GTTCCCAGCG CGGCTGTGGC TGCACCCAAT TCCAGGTGGG AACGCTGCGA AATTCCAGTG

       490        500        510        520        530        540
TCATATGCCG CAATCGACAT GATTGTGAAG CTCTGGTTTG TGCTGTTGGC ACCCGTCGTG

       550        560        570        580        590        600
CTGATTATGG CTGTGATCAT TCAATCTTCC TATCATCGTG ATCGGGAGAG GATCTGGTAC

       610        620        630        640        650        660
TATGCCAGAC GTGTGTTCAT GTTCTACACG GCCTGCTTTG TCATGATGGT GCCTTATTAC

       670        680        690        700        710        720
TTCGTCAGAG TCATGCTGAG CGACTTTGCT TTGGTTGATA TAAAAACAAA AACGGCGAAC

       730        740        750        760        770        780
AGCGACGGTT GTGATTCGAC ATTTCTTGAT TATCTGAACA TGTTCACTCA CGTGATTTAC

       790        800        810        820        830        840
AGTTTTAAGT TGGTGGTGTT TGCTTTGTTC ATTGTCCTGT TTTGCTCCAT AAACCCGATG

       850        860        870        880        890        900
GAAACGCTGG AAGAATGCTT GGAGAGGGCC GATGCTGAGA GGCAAAGTCG GTCAGAAGCA

       910        920        930        940        950        960
TCCCAGGGTG AAAGGAGGCT GCCAATCAAC ACATGCTGTA TAAAGTTGAT TGAATTGATA

       970        980        990       1000       1010       1020
AAGCAGTATG TAAGCACTCT CTCTAAAGCC ACGAGGGACA ATTCTGGCGA AAGGGCCAAT

      1030       1040       1050       1060       1070       1080
TTGCCAGAGA ATGCTGAAGA TATTGGAACA ACTGGCAGTG ATCAGCTACC GACTGAGGTC

      1090       1100       1110       1120       1130       1140
ACCGTGACCC CAAATTCATC GGCTGTGTTT AGCACTGGAG GAACGGTGTC TCCAGTCTAA

      1150       1160       1170       1180       1190       1200
.......... .......... .......... .......... .......... ..........
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FIG. 8